RNA-seq Transcriptional Profiling of an Arbuscular Mycorrhiza Provides Insights into Regulated and Coordinated Gene Expression in *Lotus japonicus* and *Rhizophagus irregularis*

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Abbreviations: AM, arbuscular mycorrhiza, AM; B&D, Broughton and Dilworth; CRP, cysteine-rich peptide; CYP, cytochrome P450; dat, days after transplanting; DEFL, defensin-like peptide; DEG, differentially expressed gene; dpi, days post-inoculation; FDR, false discovery rate; Gloin1, *Rhizophagus irregularis* genome assembly; GLP, germin-like protein; GO, gene ontology; GPAT, glycerol-3-phosphate acyltransferase; Lj2.5, *Lotus japonicus* genome assembly build 2.5; LTP, lipid transfer protein; NCR, nodule-specific cysteine-rich; Pi, orthophosphate; RN, root nodule; RPKM, reads per kilobase per million reads; VAMP, vesicle-associated membrane protein.

Footnotes: Data sets of short reads have been deposited in the DNA Data Bank of Japan Sequence Read Archive under the accession number DRA000535, DRA001845 and DRA002581.

Abstract

Gene expression during arbuscular mycorrhizal development is highly orchestrated in both plants and arbuscular mycorrhizal fungi. To elucidate the gene expression profiles of the symbiotic association, we performed a digital gene expression analysis of *Lotus japonicus* and *Rhizophagus irregularis* using a HiSeq 2000 next-generation sequencer with a Cufflinks assembly and *de novo* transcriptome assembly. There were 3,641 genes differentially expressed during arbuscular mycorrhizal development, approximately 80% of which were upregulated. The upregulated genes included secreted proteins, transporters, proteins involved in lipid and amino acid metabolism, ribosomes, and histones. We also detected many genes that were differentially expressed in small-secreted peptides and transcription factors, which may be involved in signal transduction or transcription regulation during symbiosis. Co-regulated genes between arbuscular mycorrhizal and root nodule symbiosis were not particularly abundant, but transcripts encoding for membrane traffic-related proteins, transporters and iron transport-related proteins were found to be highly co-upregulated. In transcripts of arbuscular mycorrhizal fungi, expansion of cytochrome P450 was observed, which may contribute to various metabolic pathways required to accommodate roots and soil. The comprehensive gene expression data of both plants and arbuscular mycorrhizal fungi provide a powerful platform for investigating the functional and molecular mechanisms underlying arbuscular mycorrhizal symbiosis.

Key words: Arbuscular mycorrhiza · *Lotus japonicus* · *Rhizophagus irregularis* · Root nodule · Symbiosis · Transcriptome

Introduction

Plant–microbe symbioses are established through a complex and highly coordinated gene network of both the plant and the microbe. Among the plant–microbe interactions, arbuscular mycorrhizas (AM) are symbiotic associations between plants and symbionts of AM fungi that belong to the phylum Glomeromycota (Schüßler et al. 2001). Their system of symbiosis is considered to be ancient on the basis of fossil records of the early land plants and analyses of the molecular clock of AM fungi (Simon et al. 1993; Remy et al. 1994; Taylor et al. 1995; Redecker et al. 2000). Currently AM symbiosis is found in the majority of land plants (Brundrett 2009). AM fungal hyphae attach to the plant root surface and traverse the epidermis through the pre-penetration apparatus, a host-derived, tunnel-like structure formed in epidermal cells (Genre et al. 2005; Genre et al. 2008). The hyphae spread into the intercellular space in the root cortex and further penetrate into inner cortical cells to develop a highly branched structure termed an arbuscule, where nutrient exchange occurs between the plant and the fungus (Bonfante and Perotto 1995; Harrison 1999).

A key step in AM symbiosis is the recognition of signal molecules between the host plant and the AM fungus. The plant roots release strigolactones into the soil, and the AM fungus recognizes the molecules that promote germination of spores, branching of hyphae, and activation of the metabolism (Akiyama et al. 2005; Besserer et al. 2006; Besserer et al. 2008). On the other hand, the AM fungus releases soluble molecules such as chitooligosaccharides (CO) (Genre et al. 2013) and lipochitooligosaccharides (LCO), which have structural characteristics similar to those of Nod factors produced by rhizobia (Maillet et al. 2011). The CO and LCO signaling molecules are likely to be recognized by the plant LysM receptor-like kinases NFR1/LYK3/CERK1 and NFR5/NFP like proteins, which trigger the signaling pathways in plant cells that are required for AM symbiosis (Op den Camp et al. 2011; Miyata et al. 2014; Zhang et al. 2015). The signal transduction mechanism in the early phase

of symbiosis has been analyzed extensively in model legume plants. Several genes are found to be necessary for the formation of both AM and root nodules (RN). The signaling pathway involving these genes was recently designated the common symbiosis signaling pathway (Kistner and Parniske 2002; Parniske 2008) and was shown to comprise leucine-rich repeat receptor kinases (Endre et al. 2002; Stracke et al. 2002), cation channels (Ané et al. 2004; Imaizumi-Anraku et al. 2005), nucleoporins (Kanamori et al. 2006; Saito et al. 2007; Groth et al. 2010), calcium and calmodulin-dependent kinases (Lévy et al. 2004; Mitra et al. 2004; Tirichine et al. 2006), and CYCLOPS/IPD3 (Messinese et al. 2007; Yano et al. 2008). These findings provide strong support for the hypothesis that a part of the early signaling pathway in RN symbiosis is recruited from the more ancient genetic system of AM symbiosis (Parniske 2008). Recently, the GRAS transcription factors NSP1 and NSP2, which were initially identified as being required for RN symbiosis, have been shown to play roles in the common symbiosis pathway (Liu et al. 2011; Maillet et al. 2011; Lauressergues et al. 2012; Delaux et al. 2013; Takeda et al. 2013). In *Medicago truncatula*, the novel GRAS transcription factor RAM1 has been shown to be indispensable for Myc factor signaling but not for Nod factor signaling (Gobbato et al. 2012). The *RAM1* gene regulates the transcript level of *RAM2*, which codes for a glycerol-3-phosphate acyltransferase that enhances cutin production to promote fungal hyphopodia formation on the root surface (Wang et al. 2012).

AM are not accompanied by clear *de novo* organ formation, unlike RN, which exhibit nodule organogenesis during symbiosis. However, specialized hyphal structures known as arbuscules are formed within the cortical cells of roots. Arbuscule development proceeds with at least five distinct stages: formation of a pre-penetration apparatus, fungal cell entry, formation of a birdsfoot-like structure (trunk hyphae), maturation of arbuscules, and arbuscule collapse (Gutjahr and Parniske 2013). The processes preceding the formation of a birdsfoot-like structure are known to require the common

symbiosis genes *CCaMK* (Demchenko et al. 2004) and *CYCLOPS* (Yano et al. 2008). Further, it has been demonstrated that mutations of *Medicago* DELLA proteins, which are repressors of gibberellic acid signaling, abrogated arbuscule formation in cortical cells (Floss et al. 2013). In *L. japonicus*, subtilase *SbtM1* was activated in cells containing or neighboring fungal hyphae (Takeda et al. 2009). When the *SbtM1* was suppressed by RNAi, both arbuscule formation and root colonization by intraradical hyphae were arrested (Takeda et al. 2009). In addition, Vapyrin, which consists of a vesicle-associated membrane protein (VAMP)-associated protein/major sperm protein domain and an ankyrin domain, is involved in arbuscule formation through membrane trafficking processes (Feddermann et al. 2010; Pumplin et al. 2010). For mature arbuscule formation, the half-size ABCG transporters, STRs, are indispensable (Zhang et al. 2010; Gutjahr et al. 2012). It seems likely that the STRs export a substrate molecule essential for arbuscule development to the periarbuscular space, but the substrate has not been identified (Zhang et al. 2010). The importance of membrane trafficking processes in symbiotic associations have also been demonstrated by studies of the exocytotic VAMPs (Ivanov et al. 2012) and Qb-SNARE family protein LjVTI12 (Lota et al. 2013), which are required for formation of the symbiotic membrane interface of arbuscules. Maturation of arbuscules has been shown to require *RAM2* (Wang et al. 2012), serine carboxypeptidase *SCP1* (Rech et al. 2013), and ethylene response factor *ERF1* (Devers et al. 2013). Notably, an AM-inducible phosphate transporter, which is localized in the periarbuscular membrane and responsible for phosphate uptake from the periarbuscular space, is also involved in arbuscule maintenance (Harrison et al. 2002; Javot et al. 2007; Kobae and Hata 2010; Yang et al. 2012).

Transcriptome analysis using microarrays and oligoarrays have been used for identifying plant genes induced in AM symbiosis (Liu et al. 2003; Manthey et al. 2004; Frenzel et al. 2005; Güimil et al. 2005; Hohnjec et al. 2005; Deguchi et al. 2007; Gomez et al. 2009; Guether et al. 2009a; Benedito et al.

2010; Hogekamp et al. 2011; Czaja et al. 2012; Gallou et al. 2012; Gaude et al. 2012; Bonneau et al. 2013; Hogekamp and Küster 2013). In particular, these analyses are frequently performed in the model legume plants *Lotus japonicus* and *M. truncatula*, for which genome decoding has advanced. According to these analyses, a number of genes are upregulated or downregulated during AM development. In particular, the genes for plant lectins, blue copper proteins, peptide transporters, proteases, annexin, glutathione *S*-transferases, and Myb transcription factors have been identified as being expressed specifically in AM. Additionally, genes that are involved in nutrient transport have also been identified, such as those for phosphate and ammonium transporters (Guether et al. 2009a; Benedito et al. 2010). Moreover, in a study that detailed the expression profiles of an infected cell analyzed by laser microdissection, it was demonstrated that the expression of genes for plant transporters, transcription factors, and lipid metabolism are regulated in arbusculated cortical cells (Gomez et al. 2009; Guether et al. 2009a; Hogekamp et al. 2011; Gaude et al. 2012 ; Hogekamp and Küster 2013). In contrast to this fairly extensive analysis of the plant side of symbiosis, transcriptome analysis of AM fungal genes involved in mycorrhizal formation has been limited. Recently however, many gene repertoires have been clarified by EST analysis and the genome sequencing project of AM fungus *Rhizophagus irregularis* DAOM197198 (Tisserant et al. 2012; Tisserant et al. 2013; Lin et al. 2014).

High-throughput sequencing technologies rapidly grow in use for whole-transcriptome analyses of gene expression. In AM researches, the technologies have been used for expression analyses of gibberellin biosynthesis genes (Takeda et al. 2015) and transcription factors (Xue et al. 2015) during AM development. However, comprehensive information of transcripts expressed in AM roots is limited. In this study, we performed RNA-seq analysis of AM roots using a next-generation sequencing system that can identify and quantify transcripts without prior knowledge of a particular gene, and then we

clarified the expressed gene profile of *L. japonicus* simultaneously and comprehensively with *R. irregularis* transcripts during AM symbiosis. Although high-quality draft genome sequences have already been produced in the genome sequencing projects of *L. japonicus* (Sato et al. 2008) and *R. irregularis* (Tisserant et al. 2013; Lin et al. 2014), the complete genomic sequences currently remain unknown. Therefore, we performed *de novo* transcriptome assembly using short read sequences from AM roots produced using a next-generation sequencer. Moreover, by comparison with the expressed gene profile of *L. japonicus* during RN formation, genes that function in the development and maintenance of both AM and RN in the late stage of the symbioses were identified.

Results and discussion

RNA-seq using Illumina HiSeq 2000

A flow chart of the RNA-seq analysis is shown in Figure 1. High quality reads produced by the Illumina HiSeq 2000 were mapped against the *L. japonicus* genome assembly Lj2.5 using the TopHat program. Approximately 64% of the high quality reads of all samples were uniquely aligned (Table 1, Supplementary Table S3). For the mapped reads, 80% to 85% were assigned to known genes that are annotated in Lj2.5, whereas the remaining reads were assigned to the uncharacterized regions in the *L. japonicus* genome. The sequences of the uncharacterized regions may include protein coding genes without prior annotation, untranslated regions, and noncoding RNAs. In our RNA-seq analysis, unmapped reads against Lj2.5 accounted for approximately 36% of the high quality reads, and likely included transcripts in the unsequenced regions of Lj2.5. In particular, we noted that the unmapped reads of AM root samples also contained *R. irregularis*-derived sequences, because oligo-dT beads were used to purify eukaryotic mRNA during the library construction. When the unmapped reads of AM roots were mapped against the *R. irregularis* genome assembly Gloin1, 2.9% of the high quality reads

were mapped (Table 1, Supplementary Table S4). The proportion of *R. irregularis*-derived reads was similar to that in a previous study (Tisserant et al. 2013).

Detection of novel transcripts using short read sequences of *L. japonicus*-Cufflinks assembly

A number of the high quality reads were assigned to uncharacterized regions in Lj2.5 (Table 1). To identify novel transcripts in the uncharacterized regions, we performed transcript assembly using the Cufflinks pipeline (Trapnell et al. 2010). After the high quality reads were mapped against Lj2.5, the mapped reads were assembled using the Cufflinks program. The assembled contigs of each sample were merged using the Cuffmerge utility to produce a union of transcripts from all samples, which yielded 125,948 contigs including a number of splice variants. To detect novel transcripts, the Cufflinks-assembled sequences were compared with the Lj2.5 annotation using Cuffcompare and closestBed. After selecting major isoforms by Cuffcompare, we found 11,766 contigs that did not overlap with the known sequences at any base, and these were considered as potentially novel transcripts in Lj2.5. To identify protein-coding genes from the assembled contigs, we predicted protein sequences using the TransDecoder in the Trinity program package. In this way, we identified 8,320 novel protein-coding genes of *L. japonicus* from the Cufflinks-assembled contigs and assigned each gene a TCONS number (Fig. 1, Supplementary Table S3).

Detection of novel transcripts using short read sequences-*de novo* transcriptome assembly

In our RNA-seq analysis, a number of the high quality reads were unmapped against Lj2.5 and Gloin1 (Table 1). Most of the reads were probably derived from sequences of *L. japonicus* and *R. irregularis* that had not been sequenced by the genome projects. To identify the novel transcripts of *L. japonicus*, the unmapped reads against Lj2.5 were *de novo* assembled using the Trinity program for the

respective samples of AM roots, nonmycorrhizal roots with or without orthophosphate (Pi) application, nodulated roots and control roots without rhizobium inoculation (Fig. 1). After pooling the *de novo* assembled contigs of all the samples, identical contigs were eliminated using CD-HIT-EST program. To extract novel transcripts from the nonredundant contigs, the sequences that showed similarity to the predicted coding sequences in Lj2.5, including the TCONS, were removed by a BLASTN search. The longest isoform in a unique gene "comp" was selected from the *de novo* assembled contigs, which consisted of 46,602 contigs, including transcripts of *L. japonicus* and *R. irregularis*. To extract plant-derived sequences, the contigs were redivided into AM roots and the other root samples. The contigs derived from root samples other than AM roots were considered to have originated from *L. japonicus*. For AM root samples, the contig sequences were analyzed by BLASTX against the nr protein database to extract plant-derived transcripts. As a result, 1,145 contigs that showed high similarity to plant sequences were identified as novel transcripts of *L. japonicus*. In total, 24,019 contigs were identified as potentially novel transcripts of *L. japonicus* by the *de novo* transcriptome assembly, and these included 13,340 putative coding sequences according to TransDecoder analysis (Fig. 1, Supplementary Table S3).

In AM roots, the *de novo* transcriptome-assembled contigs contain *R. irregularis*-derived transcripts as well as plant-derived transcripts. If *R. irregularis* transcripts could be extracted from the *de novo* transcriptome assembly of AM roots, it would be useful not only for transcriptome analysis of other AM fungal species for which whole genome sequencing has not been performed, but also for understanding of the molecular mechanism underlying AM development. Therefore, we tried to extract *R. irregularis* transcripts from the *de novo* assembly of AM roots. When the plant-derived transcripts detected in the above analysis were removed from the *de novo* assembled contigs of AM roots, 22,583 contigs were left as transcripts in which *R. irregularis*-derived contigs were enriched (Supplementary

Table S5). To evaluate how the *de novo* assembled contigs covered sequences of *R. irregularis*, the contigs were compared with the nr protein sequences using BLASTX (E-value cutoff = 10^{-10}). Approximately 67% of the contigs showed high similarity to sequences with prior annotation in the *R. irregularis* genome, whereas the contigs that occurred in the BLAST top hit species other than *R. irregularis* made up less than 5% of the total (Fig. 2A), indicating that the contamination of transcripts from other organisms was minimized. However, the other 29% of contigs showed no significant similarity to the nr database. The no hit sequences may contain novel transcripts of *R. irregularis* as well as some misassembled sequences and contaminated sequences. The *R. irregularis* transcripts detected in the *de novo* assembly were also characterized by the expression levels: there were quite a few transcripts having low gene expression levels (Fig. 2B). This means that fungal transcripts expressed at very low levels have not been assembled by the *de novo* assembly, because the fungal short reads are very scarce in AM roots. The results of these analyses indicate that the *de novo* transcriptome assembly is useful for unveiling the outline of expressed gene repertories in AM fungi whose genome sequence has not been determined, although the method requires attention to some contaminated sequences and is not suitable for the detection of AM fungal transcripts with low level expression.

Gene expression profiling during AM development in *L. japonicus*

To identify differentially expressed genes (DEGs) of *L. japonicus* during AM development, digital gene expression profiles of AM and nonmycorrhizal roots were analyzed. For the short read mapping, we prepared a reference sequence data set that contained the *L. japonicus* genome assembly Lj2.5 and the unique *de novo* assembled contigs derived from *L. japonicus* (http://mycorrhiza.nibb.ac.jp). The expression levels of 57,103 putative protein coding sequences in the reference sequence data set were

compared between AM roots and nonmycorrhizal roots at 27 days after transplantation (dat) using the program iDEGES/edgeR. Using a false discovery rate (FDR) cut-off of 0.001, we identified 5,098 DEGs in AM roots versus nonmycorrhizal roots at 27 dat (Fig. 3A, Supplementary Table S6). The large number of DEGs may include genes related to AM development as well as genes related to the difference in plant growth between AM plants and nonmycorrhizal plants. In our cultivating system, the shoot biomass and shoot P concentration of AM plants were more than 2-fold higher than those of nonmycorrhizal plants at 4 weeks after inoculation (Supplementary Fig. S1). To compensate for the difference of plant growth, the gene expression levels of AM roots were compared with those of nonmycorrhizal roots at 15 dat, when the Pi concentration was almost equivalent to AM roots (Supplementary Fig. S1). The comparison revealed 8,453 DEGs (Fig. 3A, Supplementary Table S6), potentially including many genes that were differentially expressed at different stages of growth. Hence, we considered upregulated or downregulated genes in both comparisons as DEGs during AM development, and thus there were a total of 3,641 DEGs (6% of the putative protein-coding genes in *L. japonicus*) (Fig. 3A). The RNA-seq data showed an increased number of DEGs compared with previous microarray studies, where several hundred transcripts were differentially expressed in AM roots or arbusculated cells (Liu et al. 2003; Manthey et al. 2004; Güimil et al. 2005; Hohnjec et al. 2005; Deguchi et al. 2007; Gomez et al. 2009; Guether et al. 2009a; Hogekamp et al. 2011; Gaude et al. 2012; Hogekamp and Küster 2013). The reasons for the greater number of DEGs by RNA-Seq are likely related to the many novel genes in the reference sequence data set and the broader dynamic range of RNA-seq than microarray analysis. RNA-seq is much suitable at detecting DEGs at low expression levels (Wang et al. 2014a). A comparison of our RNA-seq data to the microarray analysis reported by Guether et al. (2009a) revealed that a large portion of DEGs (59%) by the 28 days post-inoculation (dpi) microarray data of *L. japonicus* overlapped with the DEGs detected by the

RNA-seq analysis (data not shown). For validation of the gene expression results obtained by RNA-seq analysis, we selected 19 genes for qRT-PCR confirmation. The relative expression levels by qRT-PCR were significantly correlated with fold changes of the gene expression by RNA-seq (r = 0.859, P < 0.001) (Supplementary Fig. S2).

For the RNA-seq analysis to detect DEGs during AM development, we prepared mature AM roots that were highly colonized by AM fungi and had developed arbuscule-containing cells. Therefore, the 3,641 DEGs in AM roots versus nonmycorrhizal roots were likely to include genes required for AM development, maintenance, degradation, and functioning. Moreover, the gene expression changes could have arisen in response to the secondary effects of AM fungal infection, such as improved plant nutrition. Our RNA-seq data showed that only 16% of the transcripts induced or suppressed during AM were co-upregulated or co-downregulated in Pi-sufficient roots (Fig. 3B, Supplementary Table S6). This is in line with microarray studies that showed a low overlap between Pi-induced gene expression and transcriptional change by AM fungal colonization in *M. truncatula* (Liu et al. 2003; Hohnjec et al. 2005; Hogekamp et al. 2011) and rice (Güimil et al. 2005).

In the DEGs during AM development, upregulated genes were about 4-fold more abundant than downregulated genes (Supplementary Table S2). Notably, a number of highly induced genes were found in AM roots, which include the well-known AM-specific genes *LjAMT2;2*, *LjGLP*, *LjMAMI*, *LjPT4*, *LjSCL3*, and *SbtM1*, and cysteine proteinases in *L. japonicus* (Kistner et al. 2005; Guether et al. 2009a; Gobbato et al. 2012; Wang et al. 2012); and aquaporins, chitinases, copper binding proteins, glutathione *S*-transferases, GDSL esterases/lipases, oligopeptide transporters, plasma membrane H^+-ATPases, *RAM1*, *RAM2*, serine carboxypeptidases, and serine-threonine receptor kinases in other legumes and cereals (Liu et al. 2003; Wulf et al. 2003; Manthey et al. 2004; Güimil et al. 2005; Hohnjec et al. 2005; Gomez et al. 2009; Guether et al. 2009a; Benedito et al. 2010; Hogekamp et al. 2011;

Gaude et al. 2012; Gobbato et al. 2012; Wang et al. 2012; Hogekamp and Küster 2013) (Table 2).

Some of the most highly induced genes in the AM roots were lectin-like genes (Table 2). Several studies have reported that lectin genes are expressed in arbusculated cells in *M. truncatula* (Wulf et al. 2003; Frenzel et al. 2005; Frenzel et al. 2006; De Hoff et al. 2009). MtLec5, an AM-specific lectin, was shown to be a secretory protein according to GFP fusion analysis (Frenzel et al. 2006). AM-specific lectins may be incorporated into cell walls and involved in the symbiotic interaction between legume plants and the AM fungus (De Hoff et al. 2009). Putative secretory proteins possessing a signal peptide are frequently found on the list of highly induced genes (Table 2). These proteins include the germin-like proteins (GLPs), subtilisin-like proteases, cysteine proteases, carboxypeptidases, chitinases, GDSL lipases, and uncharacterized proteins (*e.g.,* CM0432.240.r2.a, CM0460.270.r2.d and LjSGA_059858.1). Transcripts of AM-inducible proteases (subtilisin-like proteases, carboxypeptidases, and cysteine proteases) accumulate in arbuscule-containing cells or cells adjacent to fungal hyphae (Guether et al. 2009a; Takeda et al. 2009; Hogekamp et al. 2011; Hogekamp and Küster 2013). The AM-induced subtilisin-like protease SbtM1 of *L. japonicus* and the serine carboxypeptidase SCP1 of *M. truncatula*, which are secreted into the apoplastic compartment including the periarbuscular space, are related to arbuscule development or intraradical hyphal extension in roots (Takeda et al. 2009; Rech et al. 2013). Class III chitinase genes are specifically induced in cells containing developing and mature arbuscules (Bonanomi et al. 2001; Hogekamp et al. 2011). Although information about the role of the symbiotic chitinases is limited (Elfstrand et al. 2005), they may be involved in generating chitin oligomers by cleaving the fungal cell wall, and may elicit plant responses to the fungal infection (Tromas et al. 2012). GLPs, which contain a cupin protein domain, are a diverse superfamily with an as-yet-uncertain function. Several GLPs are also highly expressed in arbuscule-containing cells (Doll et al. 2003; Güimil et al. 2005; Hogekamp and Küster 2013). Given that GLPs are involved in

nonrace-specific disease-resistance in the apoplastic compartment (Breen and Bellgard 2010), AM-induced GLPs may help to regulate fungal infection in arbuscule-containing cells. The putative secretory proteins that were highly induced in AM roots may participate in the control of the symbiotic relationship in the apoplastic space via the production of signal molecules, degradation of structural molecules in plant and fungal cell walls and construction of a periarbuscular space in which nutrients and signal molecules can be exchanged between plants and fungi.

Notably, several genes related to the metabolism of fatty acids and lipids were highly upregulated in AM roots (*e.g.*, glycerol-3-phosphate acyltransferases (GPAT), palmitoyl-acyl carrier protein thioesterase, GDSL esterases, triacylglycerol lipase, and ceramidase; Table 2), which is in general agreement with previous array studies (Gaude et al. 2012). *LjFatB*, the palmitoyl-acyl carrier protein thioesterase gene, was specifically expressed in AM roots, which also concurs with previous findings (Wewer et al. 2014). Palmitoyl-acyl carrier protein thioesterases are involved in chain termination in the pathway of *de novo* fatty acid synthesis, which generate palmitic acid in plastids. In *M. truncatula* roots colonizing AM fungi, the level of palmitic acid increased 2.7-fold compared with that in noninoculated plants according to the GC-TOF-MS analysis, which was also confirmed by the increased transcript levels for key enzymes of fatty acid biosynthesis (Lohse et al. 2005). GPATs catalyze the synthesis of lysophosphatidic acid from long-chain acyl-CoA and glycerol-3-phosphate, both of which play important roles in the biosynthesis of cutins, suberins, and lipids. In *M. truncatula*, the *ram2* mutant, which has a mutation in the GPAT gene, was indispensable for the formation of fungal hyphopodia and arbuscules, suggesting that *RAM2* promotes hyphal penetration into plant cells by enhancing the production of cutin as a signal molecule (Wang et al. 2012). We conclude from these results that the metabolism of fatty acids and lipids may be implicated in the generation of signal molecules and membrane components that comprise periarbuscular membranes.

Gene ontology (GO) enrichment analysis of DEGs during AM

To characterize the DEGs during AM symbiosis, GO enrichment analysis was performed. It revealed that a large number of GO terms were over-represented (Supplementary Table S9, S10). Several GO terms e.g., *"structural constituent of ribosome*," "*translation,"* and *"ribosome"* were strongly enriched in upregulated genes during AM, which includes ribosomal protein genes (Supplementary Table S9). The eukaryotic ribosome is composed of approximately 79 ribosomal proteins. In plants, each ribosomal protein is encoded by more than one paralogous gene; *e.g.*, there are two to seven paralogous genes per ribosomal protein in *Arabidopsis thaliana* (Byrne 2009). Many of the paralogous genes are differentially expressed during development and by abiotic stresses, which may result in an altered composition of ribosomes and biased translation (Byrne 2009; Xue and Barna 2012; Wang et al. 2013). The AM-induced ribosomal protein genes detected by our RNA-seq analysis of *L. japonicus* may participate in the development of AM.

Transporter genes were also over-represented among the up-regulated genes (Supplementary Table S9). Colonization of AM fungi into roots resulted in the upregulation of transcripts encoding for plant transporters such as the phosphate transporter LjPT4, ammonium transporter LjAMT2;2, ABCG transporter LjSTR, H^+-ATPases, aquaporins, peptide transporters, potassium transporters, zinc transporters, nitrate transporters, and cation exchangers (Supplementary Table S6), similar to that reported in the previous transcriptome analysis (Guether et al. 2009a; Benedito et al. 2010; Gaude et al. 2012). AM-specific Pi transporters are localized in the periarbuscular membrane, and responsible for Pi transport from the periarbuscular space to plant cells and maintenance of arbuscules (Harrison et al. 2002; Javot et al. 2007; Yang et al. 2012). Recently, the *M. truncatula* H^+-ATPase HA1 was found to be involved in Pi uptake via the mycorrhizal pathway (Krajinski et al. 2014; Wang et al. 2014b). The HA1

was shown to be involved in acidification of the periarbuscular space, which is important for the function of the AM-specific Pi transporter, a Pi/H^+ symporter. *LjAMT2;2*, a high-affinity ammonium transporter gene belonging to the AMT2 subfamily, was preferentially expressed in arbuscule-containing cortical cells in *L. japonicus* (Guether et al. 2009o). The LjAMT2;2 protein is hypothesized to be responsible for ammonium uptake from the periarbuscular space. In contrast to the transporters that are involved in nutrient uptake, half-size ABCG transporter STRs were identified in *M. truncatula* and *Oryza sativa*, which are required for arbuscule development, although the substrate that STRs transport is unknown (Zhang et al. 2010; Gutjahr et al. 2012).

The GO terms "*chromatin"* and *"nucleosome,"* in which histone H2A and H4 were included, were enriched in AM-induced genes (Supplementary Table S9). A core histone consists of histone H2A, H2B, H3, and H4 synthesis, which occurs in the late G1 and S phases during cell division or DNA endoreduplication. During AM development, especially during formation of pre-penetration apparatus and arbuscules, a drastic cellular rearrangement occurs (Genre et al. 2005; Genre et al. 2008). In arbusculated cortical cells, plant cell nuclei undergo endoreduplication (Berta et al. 2000; Bainard et al. 2011; Gutjahr and Parniske 2013). Transcripts of histone H2A are known to be accumulated in nondividing cortical cells that undergo endoreduplication (Koning et al. 1991), indicating that the upregulated histone genes in AM roots may reflect the endoreduplication in arbusculated cortical cells (Gutjahr and Parniske 2013).

Pathways of cysteine biosynthesis via serine (cysteine synthase, serine acetyltransferase, phosphoserine aminotransferase, phosphoglycerate dehydrogenase, and serine hydroxymethyltransferase) and arginine metabolism (glutamine synthetase, alanine glyoxylate aminotransferase, argininosuccinate synthase, and arginine decarboxylase) were activated by AM fungal colonization (Supplementary Table S6, S9). According to GC-MS profiling of the amino acids in

M. truncatula roots, steady-state levels of Cys and Arg as well as Asn, Asp, Gln, Glu, Lys, Trp, and Tyr were increased by inoculation of AM fungi, and this finding was also supported by the increased expression of the amino acid biosynthetic genes (Lohse et al. 2005). The alteration in amino acid metabolism in plant root cells during AM development may reflect stimulation of protein biosynthesis by AM colonization or an increase in nitrogen supply from AM fungi (Lohse et al. 2005).

A considerable number of GO terms were significantly over-represented in downregulated genes during AM development, and these were found to include many signal transduction- and carbohydrate metabolism-related GO terms (Supplementary Table S10). In particular, there was a decreased gene expression of trehalose-phosphate synthase (Supplementary Table S6). Trehalose-phosphate synthases catalyze the generation of trehalose-6-phosphate from UDP-glucose and glucose-6-phosphate. Subsequently, trehalose-6-phosphate is dephosphorylated by trehalose-6-phosphate phosphatase and then converted into trehalose. Plant trehalose-6-phosphates have been shown to regulate sucrose utilization and starch metabolism and to modulate developmental processes (Ponnu et al. 2011). In *A. thaliana* leaves, trehalose-6-phosphate promotes activation of ADP-glucose pyrophosphorylase, which is a regulatory enzyme for starch synthesis, leading to accumulation of starch (Kolbe et al. 2005). AM fungal colonization led to the disappearance of starch from arbuscule-containing cortical cells, indicating that starch stored in the roots is broken down to supply the carbohydrates demanded by the fungus (Gutjahr et al. 2009). This result in addition to the upregulation of the amylase gene in AM roots (Supplementary Table S6), suggests that the suppression of trehalose-phosphate synthase genes during AM development is likely to be associated with starch degradation via the regulation of carbohydrate metabolism by trehalose-6-phosphate.

Co-regulated gene expression profile between AM and RN development in *L. japonicus*

The common symbiosis genes that are essential for both AM and RN symbioses are grouped into two classes: one class of genes is involved in the signal transduction pathway that is required for the initial infection by AM fungi and rhizobia, and the second class of genes is required for the intracellular accommodation of the symbionts (Gutjahr and Parniske 2013). The second class of genes includes the *Vapyrin* gene, which is responsible for epidermal cell infection and arbuscule formation by AM fungi and infection threads and nodule formation after rhizobial infection, and has been shown to be upregulated after colonization by AM fungi and rhizobia (Feddermann et al. 2010; Pumplin et al. 2010; Murray et al. 2011). This indicates that some genes induced in both AM and RN have the potential to function in the development or maintenance of the symbioses. To identify the genes co-upregulated or co-downregulated in AM and RN symbioses, the DEGs during AM development were compared with DEGs between nodulated roots and un-inoculated roots in *L. japonicus*. Hierarchical clustering analysis using reads per kilobase per million (RPKM) data of all samples showed that a single un-inoculated root sample used as a control for nodulated roots had a considerably different profile of gene expression from other samples (Supplementary Fig. S3). The un-inoculated root sample was removed from the subsequent pairwise comparison analyses. A total of 3,248 DEGs between nodulated roots and un-inoculated roots were identified (Supplementary Table S7). The DEGs contained well-known nodulin genes (Kouchi et al. 2004), including *NIN* (CM0102.250.r2.m), leghemoglobins (CM0089.1180.r2.m, CM0034.610.r2.m), and nodulins such as MtN21/EamA-like transporter (CM0005.600.r2.m, CM0337.420.r2.m), homocitrate synthase (CM0001.710.r2.m), ENOD18 (LjT37A17.60.r2.m), ENOD40 (CM0161.400.r2.m), NOD26-like intrinsic protein (CM0046.1620.r2.m), and LjNOD70 (LjB08O09.30.r2.a).

DEGs shared between AM and RN symbiosis included only 275 transcripts, or approximately 7.6%

and 8.5% of DEGs during AM and RN, respectively (Fig. 3C). Several components of signal transduction pathways, such as receptor kinases and transcription factors, were co-upregulated (Table 3, Supplementary Table S8), highlighting the possible existence of a common transcriptional reprogramming process between the two symbioses. In *M. truncatula*, a *Vapyrin* required for intracellular infection by AM fungi and rhizobia is known to be co-upregulated in both AM and RN (Pumplin et al. 2010; Murray et al. 2011). A similar expression pattern was also observed for *L. japonicus Vapyrin* (LjSGA_008026.1) (Supplementary Table S8). VAPYRIN proteins in *M. truncatula* and petunia are associated with small subcellular compartments and may promote intracellular accommodation of endosymbionts by interacting with membranes and/or with the cytoskeleton (Pumplin et al. 2010; Feddermann and Reinhardt 2011; Murray et al. 2011; Gutjahr and Parniske 2013). We found that transcripts of an exocyst complex protein Exo70 (*LjExo70I1*) were accumulated in both AM and nodulated roots (Table 3). Exo70 is a subunit of an octameric exocyst complex that consists of Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 that function in the tethering of secretory vesicles to the plasma membrane before exocytosis (He and Guo 2009). Exo70 is highly expanded in plants compared with other exocyst subunits; for example, *A. thaliana* possesses 23 genes encoding Exo70 in the genome (Synek et al. 2006). From our database of *L. japonicus* transcripts, at least 25 transcripts of Exo70 were found (Supplementary Table S3). The AM and RN-inducible *LjExo70I1* were clustered into the Exo70I clade (Supplementary Fig. S4). Interestingly, the Exo70I clade includes an *M. truncatula* Exo70 (Medtr1g017910.1), the transcript of which was accumulated in arbuscule-containing cortical cells and nodules (Hogekamp et al. 2011; Gaude et al. 2012; Seabra et al. 2012), but is lost in *Arabidopsis* species (Cvrčková et al. 2012). This finding suggests that the legume Exo70 induced in AM and RN is likely to be involved in the development of both symbioses via a vesicle trafficking-dependent process. The significance of the vesicle trafficking for AM and/or RN symbiosis

was also supported by several studies of the R-SNARE proteins (VAMP72) (Ivanov et al. 2012), the Qb-SNARE protein (LjVTI12) (Lota et al. 2013), and the exocyst complex protein (Exo84b) (Genre et al. 2012).

Several transporters known as nodulins, which are induced by rhizobial infection, also showed an increased expression in AM roots; although the gene expression levels were different between AM and RN (Fig. 3C, Table 3). For example, the *LjSen1* coding for a vacuolar iron transporter (Hakoyama et al. 2012) was highly induced in both AM and RN, whereas an EaMA-like transporter and a major facilitator superfamily *Nlj70* were preferentially expressed in nodulated roots compared with AM roots. Additional nodulin genes co-upregulated by AM fungal and rhizobial infection included the putative aquaporins *LjNIP1* (CM0046.1610.r2.m) and *LjNIP1-2* (CM0046.1620.r2.m), which are arranged in tandem along a chromosome in the *L. japonicus* genome (Table 3). However, the expression of these two aquaporins occurred in a symbiont-dependent manner: the gene expression of *LjNIP1* was more activated during AM while *LjNIP1-2* was more activated during RN, which is in agreement with previous studies (Guether et al. 2009a; Høgslund et al. 2009; Giovannetti et al. 2012). These results suggest that the co-upregulated transporter genes may be involved in common functions or developmental processes in AM and RN symbioses, and that some of the transporter genes are likely to be more highly expressed depending on the presence of the root symbiont to express cell function characteristic of each symbiosis. Furthermore, the *LjPT4* transcript, which is an AM-specific phosphate transporter gene, showed elevated levels in nodulated roots, although the expression levels were lower than in AM roots. *LjPT4* expression has been observed in root tips, presumably in root apical meristems, even in the absence of AM fungi (Volpe et al. 2013). The induction of *LjPT4* in the nodulated roots might be activated by the nodule meristems and independent of the infected region.

LjSen1 and *LjMATE1*, which are required for nitrogen fixation in nodules (Hakoyama et al. 2012;

Takanashi et al. 2013), were highly co-upregulated (Table 3). *LjSen1*, which encodes for a putative vacuolar iron transporter, is expressed specifically in infected cells of nodules and is involved in symbiosome and bacteroid differentiation as well as in nitrogen fixation (Hakoyama et al. 2012). *LjMATE1*, whose protein catalyzes the efflux of citrate, is also expressed in infected cells of nodules and is responsible for iron uptake, possibly because of the formation of a ferric citrate complex in apoplasts of the infected region (Takanashi et al. 2013). Iron is needed for the functional expression of iron-containing proteins such as plant leghemoglobin and bacterial nitrogenase in nitrogen fixing nodules, and deficiencies in iron can then affect nitrogen fixation activity and development of the nodules (Brear et al. 2013). We also found that oligopeptide transporters were also co-upregulated in both symbioses (Table 3). In the actinorhizal tree *Casuarina glauca*, several oligopeptide transporter genes are induced in actinorhizal nodules as well as in AM roots (Tromas et al. 2012). In a recent study, an oligopeptide transporter was shown to be involved in iron transport and redistribution in plants (Zhai et al. 2014). The accumulation of iron transport-related transcripts during AM and RN symbioses may be related to the extensive gene induction of the iron-containing proteins leghemoglobin and cytochrome P450 (Table 3). However, the role of the leghemoglobins and cytochrome P450s in AM symbiosis remains to be elucidated.

Expression profiling of small-secreted peptides

Some peptides secreted from host plants, such as cysteine-rich peptides (CRPs) and CLE peptides, are known to play a significant role in AM and RN development. In *M. truncatula* belonging to the inverted repeat-lacking clade, nodule-specific cysteine-rich (NCR) peptides are involved in terminal bacteroid differentiation (Van de Velde et al. 2010). By contrast, *L. japonicus* belonging to the noninverted repeat-lacking clade has no NCR peptide-like genes in the genome (Mergaert et al. 2003;

Alunni et al. 2007), which was also confirmed by our RNA-seq data. Transcripts of some CRPs, including defensin-like peptides (DEFLs) and lipid transfer proteins (LTPs), have shown elevated levels in AM roots (Benedito et al. 2010; Tesfaye et al. 2013). We identified CRP genes from the *L. japonicus* genome and the *de novo* transcriptome-assembled contigs using the SPADA computational pipeline, which is a homology-based gene predictor for CRP surveys at the genome level (Zhou et al. 2013). A total of 213 genes in 16 subfamilies of CRPs were detected (Table 4, Supplementary Table S11). Fewer genes were detected in *L. japonicus* than in *A. thaliana* and *M. truncatula* (745 and 1170 genes, respectively) (Zhou et al. 2013). This was attributed to the incomplete genome sequence information of *L. japonicus* and the loss of NCR peptide-like genes, of which there are three and 583 in *A. thaliana* and *M. truncatula*, respectively (Zhou et al. 2013). In this study, *de novo* transcriptome assembly was performed to compensate for the incomplete genome information. However, the obtained data were restricted to root samples, including AM and nodulated roots. More CRP genes are expected to be identified by analyzing the transcriptome of the aboveground plant tissues.

The expression of the predicted CRPs was analyzed using RNA-seq data. For the 213 CRPs, 27 genes were differentially expressed in AM roots compared with nonmycorrhizal roots, and most of these were upregulated (Table 4, Supplementary Table S11). In particular, several genes in the LTP, DEFL, ripening related proteins, and chitinase/hevein subfamilies were highly induced in AM roots. In nodulated roots, nine and 23 CRPs were upregulated and downregulated, respectively. The co-upregulated CRPs in AM and nodulated roots consisted of only one gene in the LTP subfamily, indicating that CRP genes are expressed by distinct regulatory mechanisms between AM and RN. Plant LTPs are thought to be involved in membrane biogenesis as well as in cutin formation, embryogenesis, defense reactions, symbiosis, and adaptation to various environmental conditions (Kader 1996). Nonspecific LTP *MtN5* in *M. truncatula* and *AsE246* in Chinese milk vetch, which are

induced by rhizobial infection, are required for nodule organogenesis and formation of infection threads (Pii et al. 2012; Lei et al. 2014). The AsE246 localizes in the symbiosome membrane and infection threads and has been shown to participate in symbiosome membrane lipid transport (Lei et al. 2014). As reported for several LTP genes (Blilou et al. 2000; Guether et al. 2009a), transcripts of the LTP genes accumulated in AM roots, but their roles in AM development are unknown.

Several DEFLs are known to be upregulated by AM fungal and rhizobial infection, although their biological function in the symbioses is unknown (Benedito et al. 2010; Tesfaye et al. 2013). Defensins are small, highly stable and CRPs that constitute a part of the innate immune system that is primarily directed against microbial pathogens, and they are an evolutionary ancient class of antimicrobial peptides present in plants, animals, and fungi (Ganz et al. 1985; Zhu 2007). In *L. japonicus*, none of the DEFLs have been annotated in Lj2.5 due to the limitations in gene modeling. Here we identified eight DEFLs of *L. japonicus* using the SPADA pipeline (Supplementary Fig. S5). Notably, *LjDef1.1-1.5* genes clustered in a DEFL subgroup (Supplementary Fig. S6) were highly upregulated in the process of AM development (Supplementary Table S12). In *M. truncatula*, transcripts of DEFLs that clustered in the subgroup belonging to the *LjDef1* genes were also accumulated in AM roots (Benedito et al. 2010; Tesfaye et al. 2013). DEFLs in the subgroup including *LjDef1* genes might have an important regulatory function in AM symbiosis of legume plants.

CLE genes encode small-secreted peptides that function as a ligand of receptor-like kinases and play a significant role in various aspects of plant growth and development (Wang and Fiers 2010). In legumes, several CLE genes are upregulated by rhizobial inoculation and they mediate long-distance signaling to control nodulation (Okamoto et al. 2009; Mortier et al. 2010; Lim et al. 2011; Mortier et al. 2011; Reid et al. 2011; Okamoto et al. 2013). Our RNA-seq analysis reconfirmed that *CLE-RS1*/*2* and *LjCLE3* genes were significantly upregulated in response to *Mesorhizobium loti* (Supplementary Table

S13). Additionally, *LjCLE16* was upregulated in nodulated roots. Interestingly, the *LjCLE16* was not induced by application of nitrate according to qRT-PCR. This result suggests that the *LjCLE16* gene is involved in regulating RN symbiosis independent of nitrogen nutrition, as seen in *CLE-RS1* (Okamoto et al. 2009). Furthermore, we analyzed *LjCLE* gene expression in AM using RNA-seq and qRT-PCR. Six *LjCLE* genes (*LjCLE7*, *LjCLE15*, *LjCLE19*, *LjCLE20*, *LjCLE24* and *LjCLE29*) were upregulated in AM roots compared with nonmycorrhizal roots (Supplementary Table S13). Notably, the expressions of *LjCLE19* and *LjCLE20* were also induced in roots by application of Pi, which concurs with a previous study (Funayama-Noguchi et al. 2011). The upregulation of *LjCLE19* and *LjCLE20* may reveal an accumulation of phosphate in plant cells by AM symbiosis. It is noteworthy that completely different CLE genes are induced between AM and RN symbiosis, implying that *L. japonicus* uses a different signaling system for regulation of AM or RN.

Expression profiling of transcription factors

Cellular reprogramming during AM development can be achieved by transcription factors (TFs). Several TF genes that are essential for the AM symbiosis have been identified from genetic analyses (Maillet et al. 2011; Gobbato et al. 2012; Delaux et al. 2013; Takeda et al. 2013). Some legume TFs are known to be up or downregulated during AM development as determined by array technology (Liu et al. 2003; Hohnjec et al. 2005; Deguchi et al. 2007; Gomez et al. 2009; Guether et al. 2009a; Hogekamp et al. 2011; Gaude et al. 2012). In our RNA-seq analysis, 1,393 transcripts were annotated as TF genes according to the criteria of PlantTFDB 3.0 (Jin et al. 2014), 120 and 118 of which were identified as DEGs during AM and RN development, respectively (Table 5, Supplementary Table S14). In particular, almost half of the members of the GRAS family were induced in AM roots, some of which are discussed below. Highly induced genes in AM were found in the AP2/ERF, C2H2, GRAS, HSF, Myb, NAC, and

NF-YC families (Supplementary Table S14, S15). The transcript *LjMAMI* belonging to Myb in *L. japonicus* was highly accumulated in arbusculated cells as well as lateral root primordia and meristems (Guether et al. 2009a; Volpe et al. 2013). Suppression of *LjMAMI* revealed that the RNAi lines of *LjMAMI* did not show any defect for mycorrhizal formation but exhibited reductions in root elongation and branching, indicating the involvement of *LjMAMI* in root growth (Volpe et al. 2013). Hogekamp et al.(2011) showed that two highly similar genes, *MtCbf1* and *MtCbf2*, both CCAAT-binding transcription regulators, are upregulated in epidermal cells of *M. truncatula* in response to the direct physical contact with AM fungi and in the cortical areas colonized by the symbionts, including arbuscule-containing cells. Our RNA-seq analysis also revealed that a homologous gene of the *MtCbfs* in *L. japonicus* (TCONS_00114768) showed upregulation in AM (Supplementary Table S15). *MtRSD*, which encodes a nodule-specific C2H2 TF in *M. truncatula*, is essential for symbiosome differentiation during RN development (Sinharoy et al. 2013). A clear induction in nodulated roots of *L. japonicus* was also found for *LjRSD* (CM0147.270.r2.m) (Supplementary Table S14). In contrast to the nodule-specific TFs, a member of the C2H2 family (CM0021.530.r2.m) was specifically expressed in AM roots. It might be anticipated that the AM-specific C2H2 TF is involved in AM development. Transcripts of a NAC TF (CM0111.130.r2.d) and a HSF TF (AM_comp12184_c0_seq1) were also specifically induced in AM roots.

Interestingly, eleven TFs of the DEGs during AM development were co-upregulated or co-downregulated in nodulated roots (Table 5, Supplementary Table S14). Among the genes co-regulated between AM and RN, four genes belonged to AP2/ERF. The ethylene response factor (ERF) required for nodulation1 (*MtERN1*) and *MtERN2*, both of which have an AP2 DNA-binding domain, have been identified as essential genes for nodule formation (Andriankaja et al. 2007; Middleton et al. 2007; Cerri et al. 2012). These two genes of the AP2/ERF family function downstream

of *CCaMK* (Middleton et al. 2007) and bind to the Nod factor-responsive regulatory unit (NF box) of early nodulin *ENOD11*, which is involved in nodule formation via control of the expression of those genes (Andriankaja et al. 2007). *MtERN1* and *MtERN2* are also induced in AM roots (Hogekamp et al. 2011). A *Lotus* AP2/ERF gene (CM0104.2670.r2.m) that shared homology with *MtERN1* and *MtERN2* was induced in nodulated roots, but was not differentially expressed in AM roots (Supplementary Table S15). Remarkably, three AP2/ERFs were strongly upregulated in both AM roots and nodulated roots (Supplementary Table S14, S15). In addition to the AP2/ERFs, GRAS (CM0139.1440.r2.d and LjSGA_020219.1) and Dof (CM0087.150.r2.d) transcriptional regulators were also highly induced in both AM and RN symbioses. These TFs might be involved in the control of gene regulatory networks shared between AM and RN.

GRAS transcriptional regulators play a significant role in early signaling in the AM and RN symbioses. Of these GRAS TFs, *RAM1* transcripts that accumulated in AM roots are known to be specifically required for AM development (Gobbato et al. 2012). In our RNA-seq data, a similar expression profile was found for a gene (CM1852.30.r2.m) encoding a homolog of the *M. truncatula* RAM1 (Supplementary Table S15). Interestingly, GRAS TF genes, CM2163.240.r2.m (*LjSCL3-1*), CM2163.230.r2.m (*LjSCL3-2*) and CM0239.240.r2.m, were also specifically induced in AM as compared with nonmycorrhizal roots but were not detected in nodulated roots. The expression patterns of the *SCARECROW-like-3* genes, *LjSCL3-1* and *LjSCL3-2*, were in good agreement with those given in a previous transcriptome study (Guether et al. 2009a). The *LjSCL3* genes form a sister group to the *LjRAM1* and the *LjDELLA* clade (Supplementary Fig. S7). In *Arabidopsis*, SCL3 plays a role in gibberellic acid signaling by interacting with DELLA protein (Zhang et al. 2011). Because DELLA functions as a regulator of arbuscule formation in *M. truncatula* (Floss et al. 2013) and in rice (Yu et al. 2014), SCL3 may control AM development mediated by gibberellic acids. The GRAS transcription

factor genes *NSP1* and *NSP2* were initially identified as genes required for nodule formation (Kaló et al. 2005; Heckmann et al. 2006; Murakami et al. 2006). Recently, however, these genes were also shown to function in mycorrhizal symbiosis (Maillet et al. 2011; Delaux et al. 2013). The expression of *Medicago NSP2* is known to be regulated by a miRNA, miR171h, which is induced by AM fungal infection, resulting in decreased abundance of *NSP2* in AM roots (Lauressergues et al. 2012). This result concurs with our qRT-PCR data for *LjNSP2* expression (Supplementary Table S15). Recently, a GRAS TF gene, *RAD1* (CM1864.540.r2.m), was found to be expressed in arbusculated cells and involved in arbuscule development in *L. japonicus* (Xue et al. 2015). The RAD1 protein interacted with RAM1 and NSP2 *in vitro* and *in vivo*. According to our criteria for detecting DEGs during AM development, *RAD1* was not found to be DEG (Supplementary Table S14). This may be because *RAD1* transcripts accumulated not only in nonmycorrhizal roots under low-Pi conditions but also in AM roots (Xue et al. 2015). In rice, a GRAS protein OsDIP1 interacted with OsRAM1 and was necessary for normal AM development (Yu et al. 2014). *OsDIP1* was upregulated in rice roots after colonization by AM fungi (Yu et al. 2014), whereas a *L. japonicus* homolog (CM0680.320.r2.m) of *OsDIP1* was not induced in AM roots in a previous transcriptome study (Xue et al. 2015) and in our study (Supplementary Table S14). Functional analyses of *DIP1* are required in legume plants.

R. irregularis transcripts in AM roots

To elucidate the gene expression profile of *R. irregularis* colonized in *L. japonicus* roots, gene expression analysis was performed by mapping short reads of AM roots against Gloin1 (Supplementary Table S4). Out of the expressed genes (RPKM > 0), 78% genes were overlapped with genes of *R. irregularis* expressed in *M. truncatula* roots (Tisserant et al. 2013). The expression levels of the genes expressed in the roots were significantly correlated between the experiments

(Supplementary Fig. S8), indicating that the gene expression profile of *R. irregularis* is not very different between *L. japonicus* roots and *M. truncatula* roots. We found that transcripts of many cytochrome P450 (CYP) genes were highly accumulated in AM roots (Supplementary Fig. S9). CYPs are heme–thiolate proteins located in the endoplasmic reticulum and catalyze the oxidation of various organic compounds such as lipids and sterols. Fungal species also have CYPs in their genomes, although the number of CYPs varies widely among these species, ranging from only 1 to over 300 (Park et al. 2008; Črešnar and Petrič 2011; Syed et al. 2014). *R. irregularis* has over 200 CYPs according to domain prediction using the InterPro database (Tisserant et al. 2013), which is a relatively large number of CYPs for a fungal species. The diversity of CYPs in the *R. irregularis* genome was evaluated to construct a phylogenetic tree of the authentic CYPs that contain both CYP signature motifs: the heme-binding sequence motif FXXGXXXCXG and the EXXR motif in the K-helix (Supplementary Fig. S9). Out of the 117 CYPs analyzed, one CYP belongs to a well-characterized CYP51 family, and the other CYPs were clustered into groups that do not include any known CYP family. Members of the CYP51 family are well-conserved housekeeping genes that participate in 14-demethylation of sterol precursors (van den Brink et al. 1998; Črešnar and Petrič 2011). Most fungal species have one or two CYP51 genes in their genomes (Moktali et al. 2012). *R. irregularis* has a single CYP51 gene that is expressed in AM roots and spores (Tisserant et al. 2013) at a similar level (Supplementary Fig. S9), indicating that the CYP51 may also function as a housekeeping gene. The function of the other CYPs in *R. irregularis* is still unknown. Some CYP groups become extensively diversified, and members of a CYP group including Gloin1_68596 are expressed in the intraradical hyphae at high levels (Supplementary Fig. S9). The CYP diversification might be related to the various metabolic processes and possibly fungal adaptation to the soil environment and host plant roots. A CYP is known to be involved in the synthesis of fatty acid and sterol (van den Brink et al. 1998; Črešnar and Petrič 2011).

One possible CYP function in *R. irregularis* may be synthesis of sterols for membrane biogenesis during arbuscule formation.

Conclusions

Here, we revealed gene expression profiles of both *L. japonicus* and *R. irregularis* during AM development by using RNA-seq analysis. In particular, potential novel transcripts of the plant and the AM fungus were identified by the Cufflinks assembly and the *de novo* transcriptome assembly. A total of 3,641 genes were differentially expressed in *L. japonicus* with *R. irregularis* infection. The DEGs included transporters, TFs, secreted peptides and proteins, fatty acid and lipid metabolism-related enzymes, amino acid metabolism-related enzymes, ribosomes, and histones. Most of the DEGs might be required for formation and maintenance of AM. We also found that 275 genes were co-regulated in both AM and RN symbioses, which included transcripts encoding for receptors, TFs, membrane traffic-related proteins, transporters and iron transport-related proteins. The co-regulated genes might function in elementary processes shared between AM and RN development. In our RNA-seq analysis, transcript information of both the plant and the AM fungus was obtained from one AM sample. This allowed us to analyze the mutual responses of both interaction partners simultaneously at the transcriptome level during AM symbiosis.

Materials and methods

Biological materials

For the transcriptome analysis of AM, one-week old seedlings of *L. japonicus* B-129 Gifu were transplanted into autoclaved river sand and supplied with half-strength Hoagland's solution containing 100 µM Pi and inoculated with 500 spores of AM fungus *R. irregularis* DAOM197198. The plants were

grown in a growth chamber (26°C, 16 h light and 8 h dark) and were harvested at 27 dpi, at which time the hyphal, arbuscular, and vesicular colonization were 54%, 51%, and 37%, respectively. Nonmycorrhizal control plants were grown for 15 and 27 dat. To produce plants with a P nutritional status similar to that of AM plants, noninoculated plants were supplied with half-strength Hoagland's solution containing 500 µM Pi from 15 dat and harvested at 27 dat. For the experiments on RN formation, *L. japonicus* seedlings were inoculated with 10^6 to 10^7 cells of *M. loti* MAFF303099 and grown in pots with autoclaved expanded clay particles supplied with N-free Broughton and Dilworth (B&D) solution (Broughton and Dilworth 1971). Nodulated roots were harvested at 12 dpi. N-deficient, uninoculated control plants in RN formation experiments were grown for 3 dat.

RNA isolation

Three biological replicates were used for the RNA-seq experiments. The whole roots were ground with liquid nitrogen using a mortar and pestle. Total RNA was isolated using a Plant RNA Isolation Reagent (Life Technologies) or an RNAiso Plus in combination with Fruit-mate (Takara Bio) following the manufacturer's instructions. Genome DNA was removed by digestion with RNase-free DNase (Qiagen) on a column, and RNA was purified and concentrated on an RNeasy column (Qiagen). Total RNA quality was evaluated by a spectrophotometer and Agilent 2100 bioanalyzer.

cDNA library preparation and sequencing

For high-throughput sequencing, sequencing libraries were constructed using a TruSeq RNA Sample Prep kit (Illumina) following the manufacturer's instructions. Fragments of about 300 bp were excised and enriched by PCR for 10 cycles. The quality of the products was checked using an Agilent 2100 bioanalyzer and quantified using a KAPA Library Quant Kit (Kapa Biosystems). Paired-end 101 bp × 2

sequencing was performed using an Illumina HiSeq 2000 instrument (Illumina). Data sets of the short reads were deposited in a DDBJ Sequence Read Archive (DRA) (accession numbers: DRA000535, DRA001845 and DRA002581).

Mapping and Cufflinks assembly

The RNA-seq reads, Phred quality score ≥ 20 in >90% of the bases, were selected as high quality reads. The reads were mapped against the *L. japonicus* genome assembly build 2.5 (Lj2.5) (http://www.kazusa.or.jp/lotus/)(Sato et al. 2008) and *R. irregularis* genome assembly (Gloin1) (http://genome.jgi.doe.gov/Gloin1/Gloin1.home.html) (Tisserant et al. 2013) using the TopHat program (Trapnell et al. 2009). The resulting data in BAM format were converted into SAM format using samtools (Li et al. 2009). Reads with low mapping quality were removed from the SAM files. The remaining reads with high mapping quality were converted into BED format using the sam2bed perl program of Xi Wang (http://bioinfo.au.tsinghua.edu.cn/member/xwang/share/sam2bed.pl). Uniquely mapped reads against Lj2.5 and Gloin1 were counted using intersectBed in the BEDTools package (Quinlan and Hall 2010).

To extract unannotated coding transcripts and intergenic expressed regions in Lj2.5, we performed a transcript assembly using the Cufflinks pipeline (Trapnell et al. 2010), which has been used for finding new genes or alternative splicing of non-model organisms by coupling with TopHat (Ward et al. 2012). First, the high quality reads mapped by the TopHat program were assembled into a parsimonious set of transcripts using the Cufflinks program. All assemblies were merged to create a single transcriptome annotation using the Cuffmerge utility (Trapnell et al. 2010). After compilation, we compared the genomic coordinates of Cufflinks-assembled transcripts to reference annotation data of Lj2.5 by Cuffcompare (Trapnell et al. 2010) and closestBed (Quinlan and Hall 2010) to identify novel transcripts.

If a Cufflinks transcript did not overlap with any known transcript, it was considered to be a putatively novel transcript and was assigned a unique number (TCONS) (Supplementary Table S3). To identify putative coding transcripts from the novel transcripts, we ran the TransDecoder program included in the Trinity software package (Garber et al. 2011). Protein sequences translated from the novel transcripts were annotated using BLASTP (E-value cutoff = 10^{-10}) against the NCBI nr protein database.

De novo transcriptome assembly

The unmapped reads of AM roots and the other root samples against Lj2.5 were assembled using the Trinity release 2012-01-25 (Garber et al. 2011) (Fig. 1). The assembled contigs were assigned a unique identifier that combines the Trinity component number (comp), the "c" designation, and the isoform number (seq) (Supplementary Table S3 and S5). After the assembled contigs derived from the two pools were merged, redundant contigs were filtered out using CD-HIT-EST (Li and Godzik 2006) with 100% identity. To remove known transcripts of *L. japonicus* from the non-redundant contigs, a BLASTN search (E-value ≤ 10^{-20}, identity ≥ 97%, score ≥ 100) was performed against the predicted coding sequences in Lj2.5 including the TCONS. The remaining nonredundant contigs derived from the nonmycorrhizal roots were novel transcripts of *L. japonicus* origin. However, the contigs derived from the AM roots were likely to contain both plant and fungal transcripts. To extract novel plant transcripts from the nonredundant contigs of AM root samples, the *de novo* assembled sequences derived from nonmycorrhizal roots were subtracted by using a BLASTN search (E-value ≤ 10^{-20}, identity ≥ 97%, and score ≥ 100). The resulting sequences were compared with the nr protein database by BLASTX search (E-value cutoff = 10^{-10}). The sequences that had high similarity with protein sequences of viridiplantae were regarded as plant-derived novel transcripts in AM roots, whereas the other sequences were likely to be enriched by *R. irregularis*-derived transcripts. For digital gene expression analysis, the longest

isoform in a unique “comp” was selected as a reference sequence.

To evaluate how the *de novo* assembled contigs enriched by *R. irregularis*-derived transcripts cover known sequences of *R. irregularis*, the *de novo* sequences were compared with annotated genes in Gloin1 (Tisserant et al. 2013) using a BLASTN search (E-value cutoff = 10^{-10}). For expression analysis, high quality short reads of AM roots were mapped against the *de novo* assembled contigs by Bowtie (Langmead and Salzberg 2012).

Annotation of small-secreted peptides and transcription factor genes

Plant CRPs were predicted from the *L. japonicus* genome and the *de novo* assembled sequences by the homology-based gene prediction program SPADA (Zhou et al. 2013) with a search E-value cutoff of 10^{-5}, using *M. truncatula* as the training model. Transcription factor genes in the *L. japonicus* genome and the *de novo* assembly were annotated using a BLASTX search against the nr protein database. The genes were further classified into transcription factor families on the basis of the Plant Transcription Factor Database v3.0 (Jin et al. 2014).

Differential expression analysis

For digital gene expression analysis, a reference sequence set of *L. japonicus* was constructed, which consisted of sequences with prior annotation in Lj2.5 and novel coding sequences predicted by the Cufflinks assembly, *de novo* assembly, and SPADA. Uniquely mapped reads against the reference sequences were counted using intersectBed in the BEDTools package (Quinlan and Hall 2010). The count of reads mapped on each transcript was normalized using the RPKM method (Mortazavi et al. 2008; Nagalakshmi et al. 2008). Hierarchical clustering analysis was performed using the normalized data of *L. japonicus* by an average linkage method. To detect DEGs of *L. japonicus* in the comparisons

of AM roots versus nonmycorrhizal roots, Pi-sufficient roots versus nonmycorrhizal roots, and nodulated roots versus un-inoculated roots, the raw counts of the digital gene expression were analyzed using iDEGES/edgeR in TCC package (Robinson et al. 2010; Sun et al. 2013). This gave an M-value (the log ratio of normalized counts between two experimental conditions for each gene), an A-value (the two-group average of the log concentrations of the gene), a *P*-value, and a FDR for each gene. Differential expression was assessed at an FDR < 0.001.

Gene ontology analysis

The BLASTP search (E-value cutoff = 10^{-10}) against the UniProt GOA database (http://www.ebi.ac.uk/GOA/) was performed to obtain the GO annotation for *L. japonicus* genes and the putative *R. irregularis* genes. The GO Terms Classifications counter tool was used to perform GO functional classification to view the distribution of gene functions at the macro level (Hu et al. 2008). The TopGO R/Bioconductor package (Alexa and Rahnenführer 2013) was used for GO functional enrichment analysis of the DEG lists using a Fisher's exact test with a weight algorithm.

Phylogenetic analysis

Protein sequences were automatically aligned using the program MUSCLE (Edgar 2004). Phylogenetic trees were generated by the neighbor-joining method using MEGA6 (Tamura et al. 2013). Bootstrap resampling was performed using 1,000 replicates.

qPCR validation of gene expression

First-strand cDNA was synthesized using a QuantiTect Reverse Transcription kit (Qiagen). Semiquantitative PCR was performed using an ABI Prism 7000 (Applied Biosystems) or a StepOne

Real-Time PCR System (Life Technologies) with a THUNDERBIRD SYBR qPCR Mix (TOYOBO) or a Power SYBR Green PCR Master Mix (Life Technologies). Primers used for the qRT-PCR are shown in Supplementary Table S1. Three independent biological replicates of each sample were used for the real-time PCR analysis. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method (Livak and Schmittgen 2001). As an internal control, the *L. japonicus* ubiquitin gene was routinely used. Melting curve analysis confirmed the single peaks.

Measurement of plant P concentration

Plants were dried at 70°C for 48 h and weighed. The shoot P concentration was determined by the ascorbic acid-molybdate blue method (Watanabe and Olsen 1965), after digestion with concentrated sulfuric acid and hydrogen peroxide at 200°C for 120 min.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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Table

Table 1 Summary statistics for the Illumina sequencing and mapping against *L. japonicus* genome assembly built 2.5 (Lj2.5) and *R. irregularis* DAOM198197 genome assembly (Gloin1)

Description	Arbuscular mycorrhizal symbiosis				Root nodule symbiosis	
	Arbuscular mycorrhizal roots (27 dpi)	Pi-sufficient, nonmycorrhizal roots (27 dat)	Pi-deficient, nonmycorrhizal roots (15 dat)	Pi-deficient, nonmycorrhizal roots (27 dat)	Nodulated roots (12 dpi)	N-deficient, uninoculated roots (3 dat)
Total raw reads	180,722,872	27,130,924	230,883,190	69,101,476	241,443,120	217,671,998
Total high quality reads	109,494,784	25,666,804	120,106,210	62,021,902	159,170,244	115,211,270
Total nucleotides (Mb)	11,058	2,751	12,130	6,202	16,076	11,636
Mapped reads against Lj2.5	61,267,448	16,454,785	75,147,775	35,039,011	109,936,109	74,648,752
Mapped to annotated genes	49,153,712	13,485,930	63,437,147	29,011,672	89,934,955	61,109,482
Mapped to uncharacterized regions	12,113,736	2,968,855	11,710,628	6,027,339	20,001,154	13,539,270
Unmapped reads against Lj2.5	48,207,336	9,212,019	44,958,435	26,982,891	49,234,135	40,562,518
Mapped reads against Gloin1	3,245,951	-	-	-	-	-
Mapped to annotated genes	2,408,124	-	-	-	-	-
Mapped to uncharacterized regions	837,827	-	-	-	-	-

Table 2 List of highly induced genes of *L. japonicus* that were upregulated over 500-fold in arbuscular mycorrhizal roots against nonmycorrhizal roots at both 15 and 27 dat

Gene ID	Log_2-fold expression changes[a]	Annotation
CM0177.220.r2.m	19.5	lectin-like protein
CM0177.230.r2.m	19.1	lectin-like protein
comp55181_c0_seq1	18.7	no hit
LjT13G24.190.r2.a	18.0	no hit
LjT15B14.160.r2.a	17.4	germin-like protein
CM2121.10.r2.a	17.2	phosphate transporter LjPT4
comp50035_c0_seq1	16.5	no hit
TCONS_00063621	16.3	PGPS/D12
comp42247_c0_seq1	16.2	no hit
TCONS_00046166	16.2	no hit
CM0021.2790.r2.m	15.7	subtilisin-like serine protease
LjSGA_016680.1	15.7	ammonium transporter LjAMT2;2
LjSGA_010083.1	15.5	class III chitinase
AM_comp243_c0_seq1	15.4	protein of unknown function
LjSGA_014720.1	15.1	ammonium transporter LjAMT2;2
CM0104.2960.r2.a	15.1	blue copper protein
comp34200_c0_seq1	14.9	no hit
CM0177.180.r2.m	14.6	lectin-like protein
CM0312.330.r2.d	14.5	UPF0497 membrane protein
CM0905.160.r2.d	14.3	glycerol-phosphate acyltransferase LjRAM2
LjSGA_088091.1	14.3	GDSL esterase/lipase
LjSGA_022237.1	14.3	germin-like protein LjGLP
CM0328.70.r2.d	14.2	palmitoyl-acyl carrier protein thioesterase LjFatB
comp3413310_c0_seq1	14.2	protein of unknown function
TCONS_00046236	14.1	no hit
LjSGA_033241.2	13.8	cysteine proteinase
comp3144752_c0_seq1	13.8	protein of unknown function
LjT13G24.120.r2.a	13.8	no hit
TCONS_00071029	13.7	no hit
LjT31N05.100.r2.d	13.7	protein of unknown function

Table 2 Cont.

Gene ID	Log_2-fold expression changes[a]	Annotation
comp2252625_c0_seq1	13.6	protein of unknown function
TCONS_00046164	13.6	protein of unknown function
CM0432.240.r2.a	13.6	protein of unknown function
LjSGA_042024.1	13.6	acidic endochitinase-like
comp3016933_c0_seq1	13.5	no hit
LjB18K24.70.r2.a	13.5	MYB-CC type transfactor LjMAMI
TCONS_00046162	13.4	no hit
LjSGA_030709.1	13.4	4-coumarate-CoA ligase
TCONS_00010809	13.3	nohit
CM0165.320.r2.d	13.3	blue copper protein
LjT29J15.40.r2.m	13.3	wound-induced protein WIN1-like
CM0284.750.r2.d	13.3	ripening-related protein
CM0046.1610.r2.m	13.1	aquaporin nip1-2
CM0042.1550.r2.m	13.0	protein of unknown function
comp1419844_c0_seq1	12.9	GDSL esterase/lipase
LjB03G07.50.r2.a	12.9	cysteine proteinase
CM0319.30.r2.m	12.9	cysteine proteinase
comp38307_c0_seq1	12.8	no hit
LjSGA_026747.2	12.8	protein of unknown function
CM1852.30.r2.m	12.8	GRAS family transcription factor LjRAM1
CM0437.340.r2.m	12.7	germin-like protein
CM1323.370.r2.d	12.7	cysteine synthase
CM0104.2930.r2.a	12.6	blue copper protein
CM0021.2780.r2.m	12.6	subtilisin-like serine protease SbtM1
LjSGA_024997.1	12.5	leucine-rich repeat receptor-like protein kinase
CM0284.730.r2.d	12.5	ripening-related protein
comp17851_c0_seq1	12.4	ubiquitin
CM2163.240.r2.m	12.3	GRAS family transcription factor
CM0337.590.r2.m	12.3	vacuolar iron transporter LjSen1
comp48150_c0_seq1	12.2	no hit
LjSGA_059858.1	12.2	citrate-binding protein-like

Table 2 Cont.

Gene ID	Log_2-fold expression changes[a]	Annotation
CM0905.50.r2.d	12.2	glycerol-3-phosphate acyl transferase
CM0437.370.r2.m	12.2	germin-like protein
LjSGA_047343.1.1	12.1	serine carboxypeptidase
CM0437.360.r2.m	12.1	germin-like protein
CM2103.230.r2.a	12.1	protein of unknown function
CM0437.380.r2.m	12.1	germin-like protein
LjT38B21.110.r2.m	12.0	protein of unknown function
CM0337.580.r2.m	11.9	ekn protein
LjSGA_033505.1	11.8	ferric-chelate reductase 1-like
CM0021.530.r2.m	11.8	palmate-like pentafoliata 1 transcription factor
TCONS_00046275	11.7	no hit
comp2336730_c0_seq1	11.7	no hit
CM0460.270.r2.d	11.7	protein of unknown function
comp2423392_c0_seq1	11.7	protein of unknown function
comp1365288_c0_seq1	11.6	alpha tubulin
LjT13G24.180.r2.a	11.6	protein of unknown function
comp1475028_c0_seq1	11.6	protein of unknown function
CM0295.990.r2.m	11.5	peptide transporter 2
TCONS_00046237	11.4	no hit
comp1458088_c0_seq1	11.4	abscisic acid 8-hydroxylase
comp1902537_c0_seq1	11.4	no hit
TCONS_00048122	11.3	U-box domain-containing protein
LjSGA_017217.1	11.3	lectin-like protein
comp3022537_c0_seq1	11.3	protein of unknown function
CM0649.280.r2.d	11.3	cysteine proteinase
CM0170.300.r2.m	11.3	hypersensitive-induced response protein
LjT47N10.60.r2.a	11.2	triacylglycerol lipase
CM0295.70.r2.d	11.2	protein of unknown function
TCONS_00041135	11.1	protein of unknown function
LjSGA_007344.1	11.1	ripening-related protein
comp2283994_c0_seq1	11.1	ribosomal 60S subunit protein

Table 2 Cont.

Gene ID	Log_2-fold expression changes[a]	Annotation
LjSGA_011183.1	11.0	late embryogenesis abundant protein 2
TCONS_00113301	11.0	no hit
LjT34E09.160.r2.m	10.9	serine-threonine protein kinase
LjSGA_107965.1	10.8	GDSL esterase/lipase
comp3106967_c0_seq1	10.8	protein of unknown function
AM_comp3523_c0_seq1	10.8	profilin
LjT13G24.110.r2.a	10.7	protein of unknown function
LjT48A12.120.r2.d	10.7	leucine-rich repeat receptor-like protein kinase
AM_comp6687_c0_seq1	10.6	protein of unknown function
LjSGA_134299.1	10.5	late embryogenesis abundant protein 2
LjT34E09.170.r2.m	10.5	protein kinase
LjSGA_093262.1	10.5	plasma membrane ATPase
TCONS_00031440	10.4	no hit
TCONS_00013079	10.4	no hit
comp1842477_c0_seq1	10.4	protein of unknown function
comp656995_c0_seq1	10.3	no hit
CM0437.160.r2.m	10.3	reticuline oxidase
AM_comp6168_c0_seq1	10.3	60S ribosomal protein
TCONS_00037845	10.3	no hit
comp3514978_c0_seq1	10.3	protein of unknown function
CM0104.2940.r2.a	10.3	blue copper protein
LjSGA_052417.1	10.2	RING-H2 finger protein
LjSGA_040047.1.1	10.2	glutathione S-transferase
CM0055.40.r2.m	10.2	CBS domain-containing protein
CM0649.270.r2.d	10.2	cysteine proteinase
LjSGA_042189.1	10.1	neutral ceramidase-like
LjSGA_003462.2	10.0	kelch-like protein 8-like
TCONS_00090005	9.9	no hit
CM0249.1340.r2.m	9.9	replication factor C
CM0021.2200.r2.m	9.3	nitrate transporter

[a]Arbuscular mycorrhizal roots/nonmycorrhizal roots (15 dat)

Table 3 Highly upregulated (> 20-fold) genes of *L. japonicus* in both arbuscular mycorrhizal and root nodule symbioses

Gene ID	Log_2-fold expression changes		Annotation
	AM[a]	RN[b]	
Transporter			
CM2121.10.r2.a	17.2	5.9	phosphate transporter LjPT4
CM0046.1610.r2.m	13.1	6.4	aquaporin NIP1-2
CM0337.590.r2.m	12.2	13.7	vacuolar iron transporter homolog 4-like LjSen1
CM0195.70.r2.d	9.3	5.5	peptide transporter PTR1-like isoform X2
CM1868.250.r2.m	8.4	7.1	nodule-specific protein Nlj70, major facilitator superfamily
LjT04I16.80.r2.d	8.3	8.7	cationic amino acid transporter
CM0165.690.r2.m	7.4	15.8	putative polyol transporter
CM0005.600.r2.m	6.8	14.5	EamA-like transporter family protein
LjB08O09.30.r2.a	6.6	14.7	nodule-specific protein Nlj70, major facilitator superfamily
CM0147.340.r2.m	6.6	15.1	MATE protein LjMATE1
CM0046.1620.r2.m	6.3	14.6	aquaporin NIP1-2
LjT10G23.80.r2.m	4.8	6.7	oligopeptide transporter
Metal ion binding			
CM0034.610.r2.m	9.6	18.2	leghemoglobin
CM0089.1180.r2.m	9.4	18.6	leghemoglobin
CM0089.1200.r2.m	6.4	16.1	leghemoglobin
Oxidoreductase			
LjSGA_008098.2	11.2	6.9	cytochrome P450
CM0229.460.r2.m	7.2	6.8	ascorbate oxidase
CM0432.2340.r2.a	5.7	7.7	cytochrome P450
Hydorolase			
LjSGA_089145.1	12.3	6.5	serine carboxypeptidase-like protein
LjSGA_047343.1.1	12.1	6.3	serine carboxypeptidase-like protein
CM0996.1250.r2.d	9.4	17.9	aspartic proteinase npenthesin
LjSGA_026427.1	8.0	5.0	class III acidic chitinase
CM0166.30.r2.m	7.0	16.1	carbonic anhydrase

Table 3 Cont.

Gene ID	Log_2-fold expression changes		Annotation
	AM[a]	RN[b]	
Synthetase			
CM0001.710.r2.m	6.8	15.3	homocitrate synthase FEN1
Transferase			
CM0393.1120.r2.d	9.2	8.0	UDP-glucosyltransferase like
Transcription factor			
CM0608.1100.r2.m	11.9	6.7	AP2 domain-containing transcription factor
CM0081.1990.r2.m	6.1	7.7	AP2 domain-containing transcription factor
Receptor kinase			
TCONS_00092329	9.2	6.2	proline-rich receptor-like protein kinase
LjSGA_079872.1	8.8	6.3	somatic embryogenesis receptor kinase
CM0244.1000.r2.m	8.1	8.3	cysteine-rich receptor-like protein kinase
LjSGA_016434.1	7.7	6.3	L-type lectin-domain containing receptor kinase
CM0166.760.r2.a	7.1	9.9	plant receptor-like serine threonine kinase
comp108286_c0_seq1	7.0	5.7	L-type lectin-domain containing receptor kinase
Others			
CM0177.220.r2.m	19.5	5.6	lectin-like protein
CM0177.230.r2.m	19.1	5.5	lectin-like protein
comp55181_c0_seq1	18.7	5.3	no hit
CM0337.580.r2.m	11.9	15.4	EKN protein
CM0170.300.r2.m	11.3	8.1	hypersensitive-induced response protein
TCONS_00031440	10.4	6.0	no hit
CM0081.520.r2.d	10.1	13.6	UPF0497, trans-membrane plant
TCONS_00082103	9.8	5.8	protein of unknown function
LjSGA_009591.1	9.1	5.2	protein of unknown function
CM0711.360.r2.m	8.6	6.4	QWRF motif-containing protein 3-like
TCONS_00110850	8.6	7.2	protein of unknown function
LjSGA_018893.1	7.6	6.9	F-box protein
TCONS_00058294	7.1	6.6	no hit
TCONS_00019018	6.7	5.7	no hit

Table 3 Cont.

Gene ID	Log_2-fold expression changes		Annotation
	AM[a]	RN[b]	
TCONS_00122984	6.6	14.8	no hit
TCONS_00102910	6.5	6.7	no hit
CM0096.900.r2.d	6.1	5.7	Exocyst complex component
comp2319704_c0_seq1	5.4	6.7	protein of unknown function
CM0221.310.r2.d	4.7	5.6	protein of unknown function

[a]Arbuscular mycorrhizal roots/nonmycorrhizal roots (15 dat).

[b]Nodulated roots/uninoculated roots.

Table 4 Differentially expressed genes (DEGs) of small-secreted peptides in *L. japonicus* by RNA-seq

Family	No. of genes	No. of DEG (upregulated – downregulated)		
		AM[a]	RN[b]	Co-regulated[c]
Defensin related	8	4 (4 - 0)	0	0
LCR/BET1 related	18	0	0	0
Ripening related protein	6	4 (4 - 0)	0	0
Rapid alkalinization factor	15	1 (1 - 0)	3 (0 - 3)	0
Thionin related	14	0	2 (2 - 0)	0
Root cap/late embryogenesis	2	0	1 (0 - 1)	0
Novel family	6	1 (1 - 0)	1 (0 - 1)	0
Bowman Birk inhibitor	2	0	2 (0 - 2)	0
Pollen Ole e I	16	2 (2 - 0)	7 (1 - 6)	0
ECA1 gametogenesis related	21	0	0	0
Lipid transfer protein	78	13 (11 - 2)	11 (6 - 5)	1 (1 - 0)
2S Albumin	1	0	0	0
Proteinase inhibitor II	1	0	0	0
Chitinase/Hevein	6	2 (2 - 0)	1 (0 - 1)	0
Kunitz type inhibitor	12	0	3 (0 - 3)	0
Unknow	7	0	1 (0 - 1)	0

[a]DEGs of arbuscular mycorrhizal roots against both nonmycorrhizal roots at 15 and 27 dat.

[b]DEGs of nodulated roots against uninoculated roots.

[c]Co-upregulated or co-downregulated genes between arbuscular mycorrhizal roots and nodulated roots.

Table 5 Differentially expressed genes (DEGs) of transcription factors in *L. japonicus* by RNA-seq

Family	No. of genes	No. of DEG (upregulated – downregulated)		
		AM[a]	RN[b]	Co-regulated[c]
AP2	19	5 (4 - 1)	4 (3 - 1)	3 (3 - 0)
ARF	11	2 (2 - 0)	1 (1 - 0)	0
ARR-B	10	0	0	0
B3	31	1 (1 - 0)	3 (3 - 0)	0
BBR-BPC	4	0	0	0
BES1	4	1 (1 - 0)	1 (0 - 1)	0
bHLH	118	5 (5 - 0)	11 (8 - 3)	0
bZIP	45	5 (4 - 1)	4 (3 - 1)	0
C2H2	85	8 (7 - 1)	8 (8 - 0)	0
C3H	40	9 (9 - 0)	0	0
CAMTA	4	0	0	0
CO-like	8	0	3 (0 - 3)	0
CPP	5	0	0	0
DBB	6	1 (1 - 0)	0	0
Dof	24	5 (4 - 1)	4 (3 - 1)	2 (2 - 0)
E2F/DP	6	0	0	0
EIL	4	0	0	0
ERF	128	11 (9 - 2)	17 (6 - 11)	1 (0 - 1)
FAR1	10	0	1 (1 - 0)	0
G2-like	33	2 (2 - 0)	3 (3 - 0)	
GATA	23	5 (5 - 0)	2 (1 - 1)	1 (1 - 0)
GeBP	3	1 (1 - 0)	0	0
GRAS	50	20 (20 - 0)	4 (3 - 1)	2 (2 - 0)
GRF	4	1 (1 - 0)	0	0
HB-other	14	1 (1 - 0)	3 (3 - 0)	0
HB-PHD	2	0	0	0
HD-ZIP	28	3 (3 - 0)	2 (1 - 1)	0
HRT-like	1	0	1 (1 - 0)	0
HSF	16	2 (1 - 1)	1 (0 - 1)	0

Table 5 Cont.

Family	No. of genes	No. of DEG (upregulated – downregulated)		
		AM[a]	RN[b]	Co-regulated[c]
LBD	45	5 (5 - 0)	5 (4 - 1)	1 (1 - 0)
LFY	2	0	0	0
LSD	4	0	0	0
MIKC	19	1 (0 - 1)	1 (1 - 0)	0
M-type	58	1 (1 - 0)	1 (1 - 0)	0
MYB	96	5 (3 - 2)	5 (3 - 2)	0
MYB_related	88	2 (1 - 1)	3 (1 - 2)	0
NAC	98	6 (5 - 1)	4 (2 - 2)	0
NF-X1	4	0	0	0
NF-YA	7	0	3 (3 - 0)	0
NF-YB	14	1 (1 - 0)	0	0
NF-YC	10	1 (1 - 0)	0	0
Nin-like	11	0	2 (2 - 0)	0
RAV	1	0	0	0
S1Fa-like	3	1 (1 - 0)	0	0
SAP	1	0	0	0
SBP	14	0	0	0
SRS	9	0	2 (2 - 0)	0
TALE	13	0	5 (2 - 3)	0
TCP	24	4 (4 - 0)	2 (2 - 0)	0
Trihelix	23	1 (1 - 0)	6 (6 - 0)	1 (1 - 0)
VOZ	1	1 (1 - 0)	0	0
Whirly	2	0	0	0
WOX	10	0	0	0
WRKY	76	3 (3 - 0)	2 (2 - 0)	0
YABBY	4	0	0	0
ZF-HD	20	0	4 (1 - 3)	0

[a]DEGs of arbuscular mycorrhizal roots against both nonmycorrhizal roots at 15 and 27 dat.

[b]DEGs of nodulated roots against uninoculated roots.

[c]Co-upregulated or co-downregulated genes between arbuscular mycorrhizal roots and nodulated roots.

Figure legends

Fig. 1 Flow chart for analysis of RNA-seq. Rounded rectangles and ovals show short reads and assembled contigs, respectively.

Fig. 2 Validation of *de novo* transcriptome-assembled contigs that putatively enriched *R. irregularis*–derived sequences in arbuscular mycorrhizal roots. (A) Top BLAST hit species distribution of the *de novo* assembled contigs. BLASTX was performed against the NCBI nr protein database. The number of top BLAST hits per taxon is shown on the x-axis. (B) Frequency distribution of transcript expression levels of *R. irregularis* in arbuscular mycorrhizal roots. The gene expression levels are the number of reads (RPKM) mapping to annotated genes in the Gloin1 database. Gray bars show the genes that share high homology with the *de novo* assembled contigs.

Fig. 3 Summary of differentially expressed genes (DEGs) in *L. japonicus*. (A) Venn diagram showing the overlap between the DEG sets of arbuscular mycorrhizal roots at 27 dpi (AM27) versus nonmycorrhizal roots at 15 dat (NM15) and versus nonmycorrhizal roots at 27 dat (NM27). A total of 3,641 overlapped genes were considered to be differentially expressed during arbuscular mycorrhizal development (AM). (B) Venn diagram showing the overlap between the DEG sets of the AM and Pi-sufficient roots (Pi). The DEG set of Pi was identified by comparison with the nonmycorrhizal roots at 15 dat. (C) Venn diagram showing the overlap between the DEG sets of the AM and root nodule (RN) symbiosis. The DEG set of RN was identified by comparison with un-inoculated roots. (D) Relationship of M values of the DEGs between AM and RN. Green: DEGs only in AM; magenta: DEGs in both AM and RN; cyan: DEGs only in RN. NR: nodulated roots; UR: un-inoculated roots.

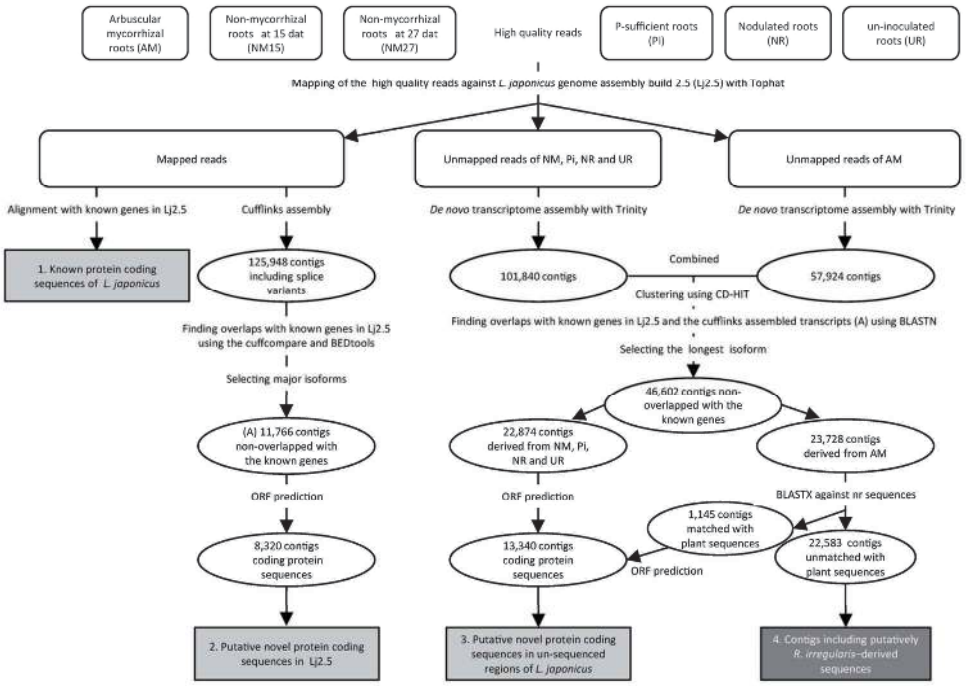

Fig. 1 Flow chart for analysis of RNA-seq. Rounded rectangles and ovals show short reads and assembled contigs, respectively.
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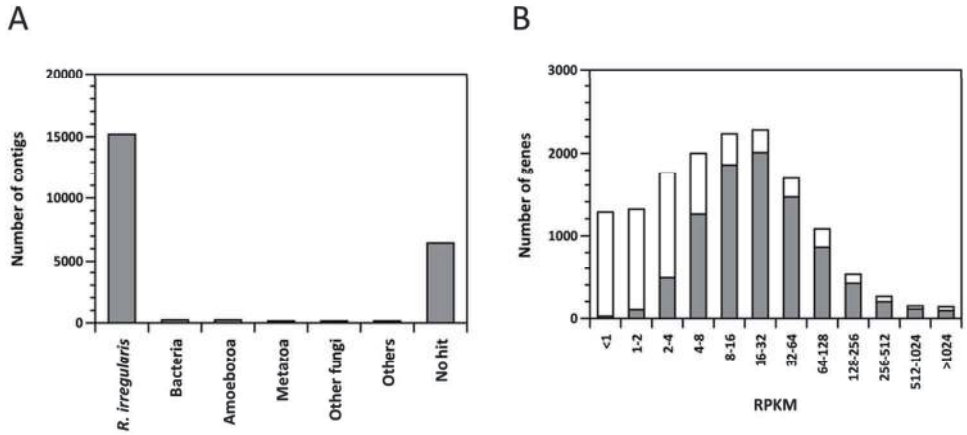

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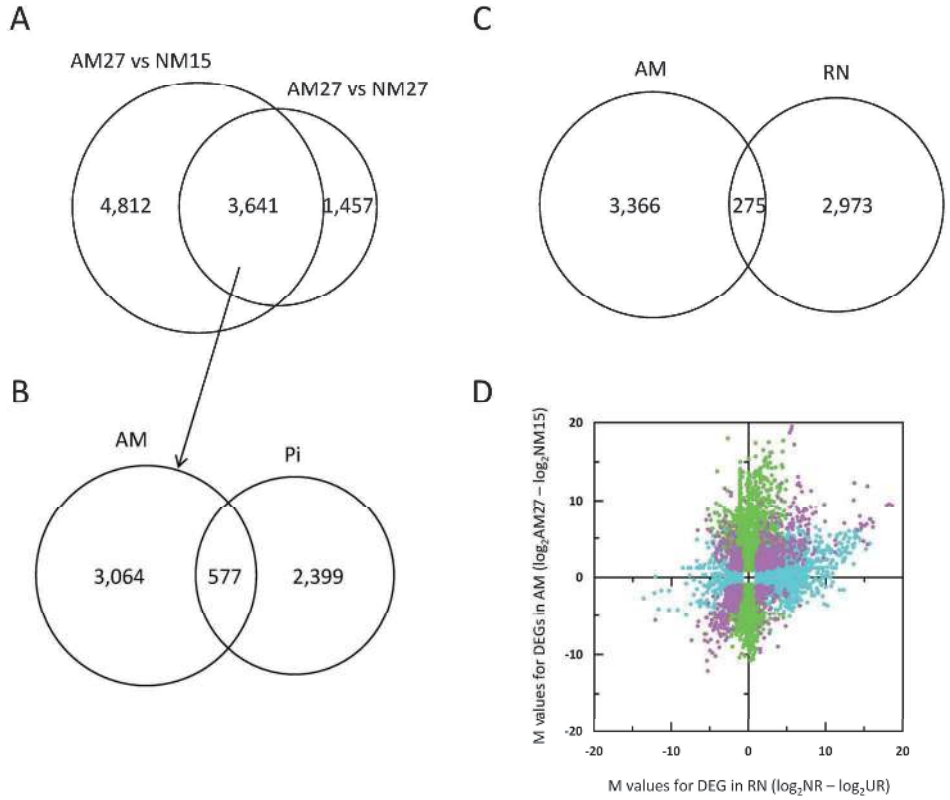

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148x124mm (300 x 300 DPI)

Supplementary Data

Supplementary Table S1 Primers used for qRT-PCR

Supplementary Table S2 Summary of differentially expressed genes in *L. japonicus*

Supplementary Table S3 Gene expression profiles in *L. japonicus* according to raw read counts and RPKM

Supplementary Table S4 Gene expression profiles of *R. irregularis* colonized in AM roots

Supplementary Table S5 *De novo* transcriptome assembled contigs in which transcripts of *R. irregularis* are enriched

Supplementary Table S6 DEGs of arbuscular mycorrhizal roots (AM) and Pi-sufficient roots (Pi) against nonmycorrhizal roots at 15 dat (NM15) or nonmycorrhizal roots at 27 dat (NM27) in *L. japonicus*

Supplementary Table S7 DEGs between nodulated roots (NR) and uninoculated roots (UR) in *L. japonicus*

Supplementary Table S8 DEGs shared between AM and RN symbiosis in *L. japonicus*

Supplementary Table S9 GO enrichment analysis of upregulated genes during AM or RN symbiosis and the co-upregulated genes in *L. japonicus*

Supplementary Table S10 GO enrichment analysis of upregulated genes during AM or RN symbiosis and the co-upregulated genes in *L. japonicus*

Supplementary Table S11 Gene expression profiles of small-secreted proteins (SSPs) in AM and RN of *L. japonicus*

Supplementary Table S12 Transcriptional changes (logFC) of defensin-like genes by RNA-seq and qRT-PCR

Supplementary Table S13 Transcriptional changes (logFC) of *LjCLE* genes by RNA-seq and qRT-PCR

Supplementary Table S14 Gene expression profiles of transcription factors (TFs) in AM and RN of *L. japonicus*

Supplementary Table S15 Transcriptional changes (logFC) of transcription factor-like genes by RNA-seq and qRT-PCR

Supplementary Fig. S1 Shoot dry weight (A) and shoot P concentration (B) of the AM plants (open circles) and nonmycorrhizal plants (closed circles) in *L. japonicus* supplied with half-strength Hoagland's solution containing 0.1 mM Pi. (C) AM colonization in *L. japonicus* roots.

Supplementary Fig. S2 Validation of the RNA-seq expression profiles by qRT-PCR. The mean transcriptional changes (log_2FC) of defensin, CLE and transcription factor-like genes between AM roots and nonmycorrhizal roots at 15 dat in Supplementary Table S12, S13 and S15 were plotted. qRT-PCR data were normalized to *LjUBQ* gene expression for each sample.

Supplementary Fig. S3 Hierarchical clustering of transcriptional profiles by the average linkage method for the normalized data (RPKM) of each sample in *L. japonicus*.

Supplementary Fig. S4 Phylogenetic analysis of Exo70.3 family in *L. japonicus*. A neighbor-joining tree based on amino acid sequences of Exo70G and Exo70I clades in the Exo70.3 family of *L. japonicus* (red) was constructed with reference sequences of *Arabidopsis thaliana* (Arath), *Medicago truncatula* (Medtr), *Oryza sativa* (Orysa) and *Solanum lycopersicum* (Solyc) (Cvrčková et al. 2012). The bar shows a genetic distance of 0.1. Bootstrap values (1,000 replicates) are shown next to the branches. The tree is rooted with ATEXO70A1 belonging to clade A in the Exo70.1 family.

Supplementary Fig. S5 Sequence alignments of defensin-like proteins of *L. japonicus* with *Vigna radiate* defensin. The sequences of *L. japonicus* were obtained from *de novo* transcriptome and Cufflinks assemblies and then confirmed by Sanger sequencing. The secondary structure of the defensins was predicted from structural information of the *Vigna radiate* defensin (PDB: 1TI5). Identical amino acids are depicted with grey lines. Connecting lines show the disulfide bonds.

Supplementary Fig. S6 Phylogenetic analysis of *L. japonicus* defensins. A neighbor-joining tree based on the defensin-like protein sequences of *L. japonicus* (red) was constructed with reference defensin sequences of *Arabidopsis thaliana* (At), *Glycine max* (Glyma) and *Medicago truncatula* (Medtr). The bar shows a genetic distance of 0.1. Bootstrap values (1,000 replicates) are shown next to the branches.

Supplementary Fig. S7 Phylogenetic analysis of the GRAS transcription factor family in *L. japonicus*. A neighbor-joining tree based on the GRAS transcription factor-like sequences of *L. japonicus* (red) was constructed with reference sequences of *Arabidopsis thaliana* (At) and *Medicago truncatula* (Mt). The bar shows a genetic distance of 0.2.

Supplementary Fig. S8 Comparison of *R. irregularis* RNA-seq data in AM roots between *M. truncatula* by Tisserant et al. (2013) and *L. japonicus* in this study. The expression level of expressed genes (RPKM > 0) of *R. irregularis* was significantly correlated between *M. truncatula* roots and *L. japonicus* roots.

Supplementary Fig. S9 Phylogenetic analysis and gene expression profiles of *R. irregularis* cytochrome P450 (CYP) genes in the Gloin1 database. A neighbor-joining tree based on the CYP protein sequences that contain the conserved motifs FXXGXXXCXG and EXXR was constructed with reference CYP sequences of *Aspergillus oryzae* (Ao), *Fusarium oxysporum* (Fo), *Fusarium verticillioides* (Fv), *Phanerochaete chrysosporium* (Pt), *Phycomyces blakesleeanus* (Pb), *Postia*

placenta (Pp), *Rhizopus oryzae* (Ro) and *Venturia inaequalis* (Vi). The bar shows a genetic distance of 0.1. A heatmap shows expression levels (log RPKM) of the *R. irregularis* CYP genes in *L. japonicus* AM roots (L), *M. truncatula* AM roots (M) (Tisserant et al. 2013) and spores (S) (Tisserant et al. 2013).