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Transcriptome Profiling of *Lotus japonicus* Roots During Arbuscular Mycorrhiza Development and Comparison with that of Nodulation

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Abstract

To better understand the molecular responses of plants to arbuscular mycorrhizal (AM) fungi, we analyzed the differential gene expression patterns of *Lotus japonicus*, a model legume, with the aid of a large-scale cDNA macroarray. Experiments were carried out considering the effects of contaminating microorganisms in the soil inoculants. When the colonization by AM fungi, i.e. *Glomus mosseae* and *Gigaspora margarita*, was well established, four cysteine protease genes were induced. *In situ* hybridization revealed that these cysteine protease genes were specifically expressed in arbuscule-containing inner cortical cells of AM roots. On the other hand, phenylpropanoid biosynthesis-related genes for phenylalanine ammonia-lyase (PAL), chalcone synthase, etc. were repressed in the later stage, although they were moderately up-regulated on the initial association with the AM fungus. Real-time RT–PCR experiments supported the array experiments. To further confirm the characteristic expression, a *PAL* promoter was fused with a reporter gene and introduced into *L. japonicus*, and then the transformants were grown with a commercial inoculum of *G. mosseae*. The reporter activity was augmented throughout the roots due to the presence of contaminating microorganisms in the inoculum. Interestingly, *G. mosseae* only colonized where the reporter activity was low. Comparison of the transcriptome profiles of AM roots and nitrogen-fixing root nodules formed with *Mesorhizobium loti* indicated that the PAL genes and other phenylpropanoid biosynthesis-related genes were similarly repressed in the two organs.

Key words: cysteine proteinase; defense response; phenylalanine ammonia-lyase; symbiosis

1. Introduction

Arbuscular mycorrhizal (AM) fungi of the phylum *Glomeromycota*[1] establish ecologically important symbiotic associations with the majority of land-plant species, allowing improved uptake of phosphate and other nutrients from the soil in exchange for plant-assimilated carbohydrates.[2,3] Additionally, AM fungi endow plants with tolerance to pathogens and abiotic stress.[4,5] In the process of colonization by AM fungi, the hyphae of extraradical mycelia branch near the host roots and form appressoria on the root surface, from which hyphae penetrate the epidermis and grow inter- and intracellularly in

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the root cortex. In the case of *Arum*-type AM (as formed in *Lotus japonicus* by *Glomus mosseae* or *Gigaspora margarita*), the hyphae of intraradical mycelia form arbuscules, which are highly branched structures thought to be the main site of nutrient exchange between the two symbiotic partners.[2,3,6] Early land-plant fossils contain structures that appear similar to arbuscules, suggesting the important role of AM fungi in the colonization of land by plants.[7–9]

In addition to AM symbiosis, leguminous plants establish a better-characterized symbiotic association with rhizobia, forming nitrogen-fixing root nodules. Recent molecular and genetic data suggest that the mechanism governing nodule formation evolved from that of AM symbiosis over time.[10,11]

The development of AM symbiosis is generally thought to accompany complex signal perception and transduction, but the understanding of the latter at the molecular level is very limited, mainly because AM fungi are obligate symbionts and the leading model plant *Arabidopsis thaliana* does not form AM roots. For a better understanding, in silico data mining,[12] the subtractive hybridization approach,[13–15] and cDNA and oligonucleotide array analyses[16–20] have been performed for *Medicago truncatula*, a model legume.[21,22] *Medicago truncatula* was also used to investigate the differential expression of chitinase genes in AM colonization, nodulation, and plant–pathogen interactions.[20,23] *Lotus japonicus* is another valuable model legume.[24] For example, *L. japonicus* has been used for elucidation of the molecular mechanisms of plant–AM fungi interactions.[25] Gene expression profiling with the aid of cDNA-amplified fragment length polymorphism has also been carried out.[10]

For transcriptome analyses of host responses to AM fungi, we here made use of a large-scale cDNA array of *L. japonicus*,[26,27] carefully eliminating the effects of contaminating microorganisms in the soil inoculants. We compared the results with a gene expression profile of root-nodule formation with *Mesorhizobium loti*, finding a number of genes commonly regulated during AM symbiosis and nodule formation.

2. Materials and methods

2.1. Plant material and microorganisms

Lotus japonicus Gifu B-129 seeds were scarified, surface-sterilized with 1% NaClO, rinsed eight times with sterile water, and then spread on 0.7% water agar plates for germination. The plates were placed for a week in a controlled-environment growth chamber (Sanyo, Tokyo, Japan) with a 16-h-day and 8-h-night cycle at 25°C, and a light intensity of 260 $\mu Es^{-1}m^{-2}$ with 60% humidity.

For AM colonization, glass tubes (30 mm diameter × 120 mm length) containing 55 mL of vermiculite supplemented with 45 mL of modified Hornum nutrient solution were autoclaved before transferring the seedlings. The concentration of phosphate was reduced from 640 μM[24] to 250 μM to facilitate the colonization. The soil inoculant of *G. mosseae* (2 g/tube; a gift from K. Nagashima, Idemitsu Kosan, Tokyo, Japan) was suspended in sterilized water and then added to the tubes. For the control plants, the *G. mosseae* inoculant suspension was filtered through a 38 μm stainless mesh and the filtrate was added to the tubes. The resulting sieved carrier was free of *G. mosseae* spores but contaminated by microorganisms equivalent to those in the whole inoculum suspension. The seedlings were grown for up to 8 weeks in a growth chamber with occasional irrigation with the modified Hornum solution. For inoculation of *G. margarita* (Central Glass Co., Tokyo, Japan), large spores were picked up with forceps under a stereomicroscope, surface-treated with 0.1% NaClO for 7 min, and then rinsed five times with sterilized water. The seedlings were inoculated with the spores and grown as above. The control plants were mock-inoculated with the final rinse and then allowed to grow further. Assessment of AM colonization was carried out by the gridline intersect method[28] after staining with trypan blue.[29]

When the initial stage of AM symbiosis was examined, we modified the 'nurse pot' method,[30] as follows. Giant spores of *G. margarita* were picked up with forceps from a commercial inoculum (Central Glass Co., Tokyo, Japan), surface-treated with 0.1% NaClO, and then rinsed with sterile water. Three sterile *L. japonicus* seedlings (1-week-old) were inoculated with 500 spores in an autoclaved plastic container (11 cm diameter × 16 cm height; Takeya Chemical Co., Osaka, Japan) with a lid and then allowed to grow further. As a non-inoculated control, the final rinse of the sterilized spores was applied to sterile seedlings in another container, followed by further growth. After 2 months, freshly prepared sterile seedlings (2-week-old) were transplanted into the containers and then allowed to grow for a week. Then, roots of the younger plants were harvested from the container inoculated with *G. margarita* or the mock-inoculated container.

For root-nodule formation, *L. japonicus* seedlings were inoculated with *M. loti* Tono and then grown for 2 weeks on vermiculite supplied with nitrogen-free Broughton and Dilworth medium as described previously.[31,32] The resulting young nodules were harvested.

2.2. cDNA array analysis

Total RNA was extracted from AM roots, root nodules, or control roots using an RNeasy Plant Mini-Kit (Qiagen, Hilden, Germany). Labeling of target cDNA, hybridization of a large-scale nylon filter array with the target, washing of membranes under high-stringency conditions,

detection of radioactive images, and data mining were all carried out as described previously.[26]

2.3. Real-time RT–PCR analysis

After treating the total RNA preparation with DNase, reverse transcription was performed with oligo(dT) and Superscript II (Invitrogen, Carlsbad, CA). Real-time PCR with a real-time RT–PCR Core Kit (Takara Bio, Otsu, Japan) and a Smart Cycler system (Cepheid, Sunnyvale, CA) was carried out as described previously.[32,33] The forward and reverse primer sets and annealing temperatures (in parentheses) were as follows: 5′-CAGTGACAAAAGGTTTGGACCTAC-3′ and 5′-ATGCAGAGAGATGTTGCTGCTG-3′ (68°C) for *LjCyp2*; 5′-AACTTTATTAGTAACTTTTAG-3′ and 5′-CTTTCACATCCGAGGAAATTG-3′ (55°C) for *LjPAL1*; 5′-GCTCAGGTGGCTGCCATCGCC-3′ and 5′-GGCAGTGTGTGGTTTGTCTCG-3′ (55°C) for *LjPAL2*; 5′-AACTTTACTAG TTTCTTCAGG-3′ and 5′-TAATTCCATATTCCGCAAATT-3′ (55°C) for *LjPAL3*; 5′-GAATGCAGATCTTACCCGCTA-3′ and 5′-TTTGCTTAAATACAAAGAATG-3′ (50°C) for *LjPAL4*; 5′-GAATGCAGATCTTACCCGCTG-3′ and 5′-ATTGCATTTGCATAAATACAG-3′ (50°C) for *LjPAL5*; 5′-AACTTAACCATTTATTTTTTT-3′ and 5′-TTG TAATGTAATGTGAGATGG-3′ (55°C) for *LjPAL6*; 5′-TTGGCTAGCATCGATTCAGGA-3′ and 5′-GTCCAGGGTGGTGCTTAAGCC-3′ (50°C) for *LjPAL7*; 5′-GCTCAGGTGGCTGCCATCGCA-3′ and 5′-GGCAGGGTGTGAGTTGATTCA-3′ (55°C) for *LjPAL8*; 5′-AACTTGCCTGCCAGTTATGTT-3′ and 5′-CTCTTGTGTTTTTCTGTAGTG-3′ (55°C) for *LjPAL9*; and 5′-AGAACAGTTTGTTTGTTTGAG-3′ and 5′-CATAAAGGAGAACTTAAAGGA-3′ (55°C) for *LjPAL10*. Amplification of the β-actin gene was carried out as described previously.[33] A single amplicon of expected size, 100–300 bp, with each primer set was observed on agarose gel electrophoresis, irrespective of whether the reverse-transcribed template was from AM roots or control roots. In order to calculate the transcript level ratios, it was assumed that each PCR cycle results in exact doubling of the amounts of amplicons.

2.4. In situ hybridization

In situ hybridization of paraffin-embedded sections was carried out as described previously.[31,33,34]

2.5. Promoter-β-glucuronidase construction, hairy root transformation and histochemical analysis of L. japonicus

The 2 kb 5′ flanking region of *LjPAL1* contains a *Bam*HI site. Therefore, to amplify the region derived from genomic DNA of *L. japonicus*, forward primer 5′-ATGCGGCCGCTGACCGACAATGGTTTATGAAC TAGCC-3′ and reverse primer 5′-ATTGATCACTTAGT ATATATGATCTCTCACTTACA-3′, containing *Not*I and *Bcl*I sites, respectively, were used for PCR. The *Bcl*I end of the promoter was ligated to the *Bam*HI site 24 bp upstream of the coding sequence of the *uidA* gene for the β-glucuronidase (GUS) reporter with a nopaline synthase terminator. Then, making use of the *Sal*I sites at the ends of the intermediate construct, the promoter-GUS unit was ligated into the *Sal*I site of pHKN29,[35] which is a derivative of pCAMBIA 1300 (CAMBIA, Canberra, Australia).

Hairy root transformation with *Agrobacterium rhizogenes* LBA 1334 was performed following the protocol of Diaz and Schlaman, Leiden University, as described previously.[33,35] Transformants with green fluorescent protein (GFP)-positive hairy roots were transferred to vermiculite containing the modified Hornum solution, inoculated with the entire *G. mosseae* inoculum or sieved carrier, and then grown as described above. When nodule formation was examined, the transformants were transferred to nitrogen-free Broughton and Dilworth medium and then inoculated with *M. loti* Tono.

Detached roots were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, and then the reaction was stopped with 75% ethanol as described previously.[35] When AM fungi were re-stained, the roots were immersed in 0.02% safranin and then observed under a stereomicroscope. Quantitative assaying of GUS activity in hairy roots was performed as described previously,[35] based on the method of Jefferson *et al.*[36] GUS-stained roots were also fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM Na-phosphate buffer (pH 7.2), washed with Na-phosphate buffer, dehydrated in an ascending ethanol series (10, 30, 50, 60, 70, 90, and 100%), immersed in 50% Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) in ethanol, and then left to stand overnight. Then, they were embedded in Technovit 7100 at room temperature by adding the polymerization agent provided in the kit. Six-micrometer sections were prepared and re-stained with 0.02% safranin when necessary.

2.6. Accession numbers

The entire nucleotide sequences of cDNAs for cysteine proteinases and PALs were determined. The accession numbers for the sequences mentioned in this paper are as follows: AB300459 (*LjCyp1*), AB300460 (*LjCyp2*), AB300461 (*LjCyp3*), AB300462 (*LjCyp4*), AB283031 (*LjPAL1*), AB283032 (*LjPAL2*), AB283033 (*LjPAL3*), AB283034 (*LjPAL4*), AB283035 (*LjPAL5*), AB283036 (*LjPAL6*), AB283037 (*LjPAL7*), AB283038 (*LjPAL8*), AB283039 (*LjPAL9*), and AB283040 (*LjPAL10*).

3. Results and discussion

3.1. Setting up cDNA array experiments with AM roots

In this work, we made use of a large-scale array of cDNAs from entire seedlings, pods, roots, and root nodules of *L. japonicus*.[26,27] We grew *L. japonicus* plants with or without AM fungi in the presence of 250 μM phosphate. At that phosphate concentration, AM fungi colonized well and the effects of phosphate depletion did not need to be taken into account. Under our growth conditions, the root length colonization by *G. mosseae* was 20 and 60% at 3 and 6 weeks after inoculation, respectively. The colonization by *G. margarita* was 10 and 30–40% at 5 and 8 weeks after inoculation, respectively. It has been pointed out that fungal transcripts account for up to 12% of the entire transcripts of AM roots,[37] which sometimes complicates analysis.[10,12–15] On the other hand, since the plant materials used for our array were grown avoiding microorganisms other than *M. loti*, a nitrogen-fixing symbiont of *L. japonicus*, our array did not contain fungal genes, making the analysis of global plant gene-expression easier.

Extraction of RNA, preparation of radioactive targets, and hybridization were principally performed for two biological replicates, although in the experiments involving *G. margarita*, the procedures were carried out in duplicate for a single biological replicate. The signal intensities of array filters in each experiment were normalized as described previously.[26] When the normalized signal intensities were compared after the two independent series of experiments, the variation was found to be basically within the twofold expression ratio (Supplementary Fig. S1A), indicating the sufficient reproducibility of our experiments.

In the initial experiments, we compared the gene-expression patterns of AM roots formed with a commercial inoculant and sterile non-infected roots, as in most previous studies.[12–15,17–19] Then, we picked up statistically significantly different genes expressed in roots 6 weeks after inoculation of the whole *G. mosseae* inoculum compared with those in control roots 3 weeks after inoculation of the sieved carrier, by means of the Significance Analysis of Microarrays Program.[38] Supplementary Table S1 shows a list of the apparently up-regulated genes in AM roots. Closely related genes annotated as caffeic acid *O*-methyltransferase were most differently expressed. Lectin genes were also differentially expressed, as previously reported.[13,15,19] The up-regulation of a gene for subtilisin-like serine protease was similar to the finding of Liu *et al.*,[16] although serine carboxypeptidase genes were not listed in our experiment. The differential expression of chitinase genes was in accordance with a previous study.[23] Glutathione *S*-transferase genes were reported to be up-regulated in AM roots.[12–14,20] In our experiment, a gene for glutathione *S*-transferase (GNf044a01) was also up-regulated by 1.61- and 2.51-fold 3 and 6 weeks, respectively, after inoculantion (not included in the supplementary table). The expression levels of blue copper protein genes[12,13,18,20] varied from experiment to experiment under our conditions (data not shown). Overall, the data in Supplementary Table S1 are consistent with those in previous papers.[12–20]

Notably, when the *G. mosseae* inoculum suspension and sieved carrier were diluted and streaked on yeast extract/peptone/glucose plates, many colonies of contaminating microorganisms appeared, their numbers and appearances being similar to each other (data not shown). Thus, the above cDNA array analysis was performed in the constant presence of background microorganisms in the AM root material. We next filtered the *G. mosseae* inoculant suspension through a 38 μm stainless mesh, *L. japonicus* seedlings were grown in the presence of the filtrate, and then the gene expression in the resulting roots was compared with that in non-infected ones. Supplementary Table S2 shows the effects of contaminating microorganisms. Genes encoding PAL, chalcone synthase and chalcone reductase, which are involved in important steps of flavonoid phytoalexin synthesis,[39] and WRKY transcription factors, which are mainly involved in tolerance to pathogen-related stress,[40] were remarkably induced. The genes annotated as caffeic acid *O*-methyltransferase, and those for chitinase and glutathione *S*-transferase were also induced. Therefore, the results in Supplementary Table S1 represent superpositioning of the effects of the AM fungus and contaminating microorganisms in the inoculant. It is noteworthy that commercial AM fungus inoculants have been used easily in a number of investigations on plant gene expression in AM roots.[12–15,17–19] Care must be taken regarding contamination in nurse plants used for inoculation of the AM fungus.[10] On the other hand, in the works of Liu *et al.*[16,20] and Salzer et al.[23] on *M. truncatula*, Guimil *et al.*[41] on rice, and ours on *L. japonicus* and *G. margarita* (see below), aseptic spores of AM fungi were inoculated into plants, making the populations of contaminating microorganisms, if any, similar between AM roots and control roots.

3.2. Expression profiling of up- and down-regulated plant genes after colonization by AM fungi

In order to subtract the above-described effects of contaminating microorganisms, we compared the gene expression patterns of AM roots inoculated with the whole *G. mosseae* inoculum and control roots inoculated with the sieved carrier only. When the average intensities on duplicate determination of gene expression were compared, the patterns indicated a significant difference in gene expression (Supplementary Fig. S1B). AM-enhanced genes were first identified after colonization by *G. mosseae* and *G. margarita* (Table 1) because they have attracted

Table 1. Up-regulated genes in *L. japonicus* roots after establishment of symbiosis with *G. mosseae* and *G. margarita*

Current annotation	Gm3/SC3	Gm6/SC3	Gi8/gni3	Gene ID	Max RE	e-value
Amino acid and nitrogen metabolism						
Asparagine synthetase	1.3	4.0	1.7	MWL032c11_r	499	2E−39
Asparagine synthetase	1.2	4.0	1.7	GNf053e06	1005	3E−49
Asparagine synthetase	0.9	3.3	1.8	MWM233f05_r	460	3E−36
Asparagine synthetase	1.1	2.4	1.4	GNf021f11	569	4E−81
Carbon metabolism						
Alpha-mannosidase	1.4	3.6	2.7	MPDL053f07_f	217	
Alpha-mannosidase	1.3	3.2	1.8	MPDL018e02_f	232	
Putative alpha-mannosidase	1.6	3.1	1.9	MPDL061d01_f	313	1E−09
Secondary metabolism						
Chalcone reductase	1.4	4.1	3.0	GNf040a09	231	6E−06
Chalcone reductase	0.7	2.1	2.3	GNf086d03	160	5E−53
Flavonoid 3-*O*-galactosyl transferase	1.1	2.0	3.4	MPD011f01_f	158	2E−29
Transport/membrane						
Plasmamembrane intrinsic protein	2.3	2.8	0.8	MWL070f05_r	1440	9E−56
Putative nitrate transporter	0.9	2.2	2.6	MWM134h03_r	88	2E−07
Aquaporin1	1.1	2.1	1.1	MWM132a07_r	208	5E−66
Plasma-membrane intrinsic protein	2.0	2.1	0.7	MWM091f06_r	1490	5E−13
Tonoplast intrinsic protein	2.3	2.1	1.7	MWM074b06_r	444	2E−42
Nodulin						
Nodulin 26-like protein	1.1	2.7	2.3	MWM104a10_r	174	5E−09
Nodule-enhanced sucrose synthase	1.3	2.3	1.2	MWL080e04_r	797	2E−78
Signal transduction						
Annexin	1.2	3.2	2.8	MPD097d02_f	135	3E−36
Annexin	1.0	2.4	2.5	MPD065b05_f	215	7E−36
Annexin	1.0	2.2	2.6	MPD042e01_f	185	2E−62
ANTI-H(O) lectin (LTA)	1.8	2.0	7.0	MWM231h03_r	24488	4E−39
Protein fate						
Cysteine proteinase (LjCyp4)	3.8	19.7	8.0	GNf089d01	664	6E−40
Cysteine proteinase (LjCyp1)	1.9	10.6	3.3	GNf032f12	251	
Cysteine proteinase (LjCyp2)	1.7	5.0	2.3	GNf037h07	964	6E−23
Cysteine proteinase (LjCyp3)	1.3	4.0	2.3	GNf071h01	226	6E−12
Cell wall						
Yieldin precursor	1.0	4.3	1.9	MWM140d02_r	75	3E−16
Pathogen-related						
Putative disease resistant protein	1.6	2.0	1.2	MPDL019h09_f	980	5E−15
Phytohormone-related						
Jasmonic acid 2	1.4	6.0	4.6	MWL076b07_r	149	7E−73
Other enzyme						
Nicotianamine synthase	4.7	5.3	2.1	GNf070f09	316	5E−21
Other category						
Dehydrin 3	2.2	8.0	6.9	MR001a01_f	228	0.0002

The data in the Gm3/SC3 and Gm6/SC3 columns are the gene expression levels in roots 3 and 6 weeks, respectively, after inoculation of the whole *G. mosseae* inoculum relative to those in control roots 3 weeks after inoculation of the sieved carrier. Since prolonged cultivation with low concentrations of phosphate may cause stress,[18] we do not think that there is any problem with the use of younger control roots. The data in the Gi8/gni3 column are the gene expression levels in roots 8 weeks after inoculation of NaClO-treated *G. margarita* relative to those in roots 3 weeks after mock-inoculation. MaxRE is the highest normalized expression level in the experiments. Genes that match hypothetical proteins of unknown function and ones that encode proteins exhibiting no homology to thus far known ones have been omitted from this table.

more interest than repressed ones.[12–15,17,19] Genes for aquaporins, also annotated as plasma-membrane intrinsic protein, tonoplast intrinsic protein, and nodulin 26-like protein, were up-regulated in AM roots, confirming the results in several reports.[14,17,19] Annexin genes were also induced in AM roots, in accordance with Manthey *et al.*[17]

Four cysteine proteinase genes, designated as *LjCyp1-4*, were most obviously up-regulated among the AM-enhanced genes (Table 1), confirming previous reports.[10,16–18] Although there were around 20 cysteine proteinase genes on our array membrane, the expression of other genes did not change or was rather repressed in AM roots. Real-time RT–PCR showed that *LjCyp2*, a representative of the four genes, was induced only at the late stage of *G. mosseae* colonization (Fig. 1A). The expression of *LjCyp2* was also high in *G. margarita*-colonized roots at the late stage (data not shown). Our *in situ* localization revealed that the induced *LjCyp2* gene was specifically expressed in arbuscule-containing inner cortical cells of *G. mosseae*-colonized roots (Fig. 1C). The *LjCyp1* transcript showed a very similar localization (not shown) to that of *LjCyp2*. The spatial expression patterns of AM-induced genes fall into two groups. The glutathione *S*-transferase,[13] serine carboxypeptidase,[16] annexin,[17] and calcium-binding protein[10] genes were reported to be expressed not only in arbuscule-containing cells but also in the cells around them. In contrast, the endoglucanase (MtCel1),[16] cysteine-rich antifungal protein,[19] and AM-induced phosphate transporter[33] genes were specifically expressed in cells that contained fungal arbuscules. The present study revealed that the *Lotus* cysteine proteinase genes are members of the latter group. It is noteworthy that the cysteine proteinase genes are expressed early in cells containing arbuscules just after maturation, whereas their levels are quite low in cells with very young arbuscules (Fig. 1E). The induced cysteine proteinases may be involved in the degradation of arbuscules, short-lived fungal organs,[2] since the PSORT program (http://psort.nibb.ac.jp/) predicted that they are secreted proteins. Alternatively, these proteases may stay within the cells, e.g. in vacuoles[42,43] and play important roles in remodeling of intracellular structures, cell cycle progression, protein turnover etc. It is also interesting that the four cysteine proteinase genes are exactly the same genes as those that are highly induced in early-senescent root nodules of ineffective nitrogen fixation.[27]

A promoter region of a calcium-binding protein gene of *L. japonicus* was reported to be activated during AM development.[10] We found that a cDNA for the calcium-binding protein (MWM036h04_r) is present on our array membrane. Unexpectedly, however, the mRNA level did not show significant variation under our experimental conditions. The mRNA level of the gene did not vary on root-nodule formation, either (http://est.kazusa.or.jp/en/plant/lotus/EST/cDNA.html). The promoter activity of the gene may not coincide with its transcript level.

In the present study, AM-repressed genes were also identified after colonization by *G. mosseae* or *G. margarita* (Table 2). Five *PAL* genes were repressed

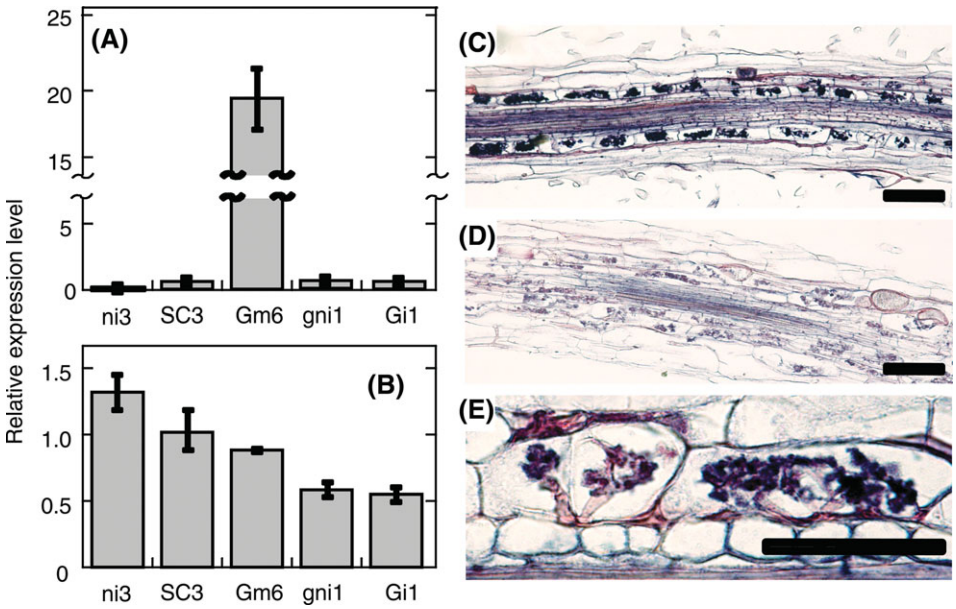

Figure 1. Expression levels and *in situ* localization of the *LjCyp2* gene. The expression levels of the *LjCyp2* (**A**) and *ß-actin* (**B**) genes relative to those in control roots 3 weeks after inoculation of the sieved carrier (SC3, level = 1) were determined by real-time RT–PCR. The means and variation of two independent experiments are shown. ni3, sterile non-infected roots grown for 3 weeks; Gm6, roots 6 weeks after inoculation of the whole *G. mosseae* inoculum; gni1, roots 1 week after mock-inoculation; and Gi1, roots 1 week after inoculation of *G. margarita* (see Materials and Methods and the legend to Table 3 for details). Longitudinal AM root sections were probed with digoxygenine-labeled antisense RNA prepared from the entire *LjCyp2* cDNA (**C** and **E**). Hybridization signals are visible as a dark blue color. When sense RNA was used as a negative control probe, much lower hybridization signals were detected except in central cylinders (**D**). Bars, 50 μm.

Table 2. Down-regulated genes in *L. japonicus* roots after establishment of symbiosis with *G. mosseae* and *G. margarita*

Current annotation	Gm3/SC3	Gm6/SC3	Gi8/gni3	Gene ID	Max RE	e-value
Amino acid and nitrogen metabolism						
Serine decarboxylase	0.24	0.17	0.06	GENf054a02	956	1E−52
Serine decarboxylase	0.30	0.24	0.11	MWM231b10_r	609	0.004
Prephenate dehydratase	0.80	0.46	0.60	MR013b06_f	153	4E−15
Carbon metabolism						
Phosphoenolpyruvate carboxylase (LjPEPC2)	0.49	0.31	0.49	MWM088d03_r	297	5E−68
UDP-glucose:protein transglucosylase	0.49	0.32	0.54	MWM177b05_r	429	3E−32
Glucose-6-phosphoate 1- dehydrogenase	0.40	0.34	0.45	GENf019d07	397	4E−23
Xyloglucan endotransglycosylase	1.00	0.37	0.11	MR065e10_f	843	1E−17
Secondary metabolism						
Phenylalanine ammonia-lyase (LjPAL3)	0.21	0.15	0.17	MR060a09_f	1262	1E−17
Phenylalanine ammonia-lyase (LjPAL4)	0.21	0.16	0.17	GENLf025c04	514	2E−38
Phenylalanine ammonia-lyase (LjPAL1)	0.25	0.24	0.17	MRL007g11_f	485	9E−38
Phenylalanine ammonia-lyase (LjPAL8)	0.31	0.25	0.26	MWL032c01_r	430	2E−40
Phenylalanine ammonia-lyase (LjPAL2)	0.27	0.26	0.22	GENLf058e04	411	0.006
Chalcone synthase	0.44	0.27	0.39	MWM170f10_r	536	8E−48
Chalcone synthase	0.28	0.36	0.63	MWM193h03_r	714	3E−80
Chalcone synthase	0.50	0.39	0.65	MWL020g05_r	450	1E−14
Deoxychalcone synthase	0.40	0.21	0.36	MWM174f04_r	485	1E−18
Chalcone reductase	0.28	0.19	0.17	MWM002d07_r	709	1E−60
Chalcone reductase	0.35	0.22	0.17	GNf090d05	728	6E−64
Caffeoyl-CoA *O*-methyltransferase	0.98	0.43	0.38	MPD011e05_f	486	7E−66
Caffeoyl-CoA *O*-methyltransferase	0.93	0.43	0.46	MWM071f11_r	240	0.00008
Isoprene synthase	1.82	0.26	0.39	MWL054c12_r	686	3E−37
Lupeol synthase	0.46	0.19	0.56	GNf046g09	309	8E−50
Transport/membrane						
Phosphate transporter (LjPT1)	0.36	0.18	0.68	MWM077d10_r	334	4E−59
Mitochondrial dicarboxylate carrier protein	1.01	0.35	0.54	MPD024c12_f	266	7E−14
Plasma membrane Ca^{2+}-ATPase	1.05	0.38	0.26	GENLf026c07	473	5E−28
Sucrose transport protein	0.40	0.39	0.53	MWM221d11_r	342	2E−47
Signal transduction						
Putative acid phosphatase	0.85	0.39	0.23	MWM048e06_r	1670	8E−24
Transcription/translation						
Transcription factor WRKY4	0.35	0.19	0.18	MWM168c07_r	690	4E−45
WRKY-type DNA binding protein	0.50	0.35	0.24	MWM240a07_r	624	5E−07
WRKY DNA-binding protein	0.36	0.35	0.31	MR083f05_f	263	0.0001
Cell wall						
Extensin-like protein	1.01	0.35	0.48	MWM170b07_r	911	0.0004
Pathogen-related						
Peroxidase	0.46	0.22	0.44	GENf076g12	384	2E−17
Peroxidase	0.47	0.37	0.76	GNf069g02	136	6E−14
Syringolide-induced protein	0.32	0.23	0.20	MWM033e05_r	745	8E−14
Syringolide-induced protein	0.35	0.29	0.39	GNf002b04	258	2E−23
Syringolide-induced protein	0.99	0.39	0.15	GNf095h04	258	6E−31
Similar to the BURP domain	0.56	0.22	0.13	MPDL062c05_f	4760	1E−48
Seed coat BURP domain protein	0.42	0.25	0.15	MPDL082d06_f	3753	9E−59

Continued

Table 2. Continued

Current annotation	Gm3/SC3	Gm6/SC3	Gi8/gni3	Gene ID	Max RE	e-value
Seed coat BURP domain protein	0.52	0.35	0.10	MPD013h01_f	4069	2E−18
PR10-1 protein	0.62	0.35	0.82	GNf017d12	1268	8E−16
Other enzymes						
Cytochrome P450-1	0.18	0.17	0.16	MR095g09_f	678	1E−19
Mannan endo-1,4-beta-mannosidase	0.55	0.22	0.59	MWM099c01_r	304	3E−57
Soluble inorganic pyrophosphatase	0.75	0.35	0.28	MPD001e10_f	603	8E−07
Cytochrome P450, putative	0.38	0.30	0.28	MR076b02_f	233	4E−10
Glutathione *S*-transferase	0.30	0.35	0.85	MWM067e12_r	1452	3E−33
Cytochrome P450 82C1	1.28	0.36	0.51	MR061f02_f	219	3E−35
ATP synthase 9	0.24	0.20	0.21	MWM223c10_r	456	3E−22
Other category						
Putative acyl-CoA oxidase	0.26	0.18	0.19	MWM217b02_r	628	1E−42

The data in the Gm3/SC3 and Gm6/SC3 columns are the gene expression levels in roots 3 and 6 weeks, respectively, after inoculation of the whole *G. mosseae* inoculum relative to those in control roots 3 weeks after inoculation of the sieved carrier. Since prolonged cultivation with low concentrations of phosphate may cause stress,[18] we do not think that there is any problem with the use of younger control roots. The data in the Gi8/gni3 column are the gene expression levels in roots 8 weeks after inoculation of NaClO-treated *G. margarita* relative to those in roots 3 weeks after mock-inoculation. MaxRE is the highest normalized expression level in the experiments. Genes that match hypothetical proteins of unknown function and ones that encode proteins exhibiting no homology to thus far known ones have been omitted from this table.

most drastically after colonization by *G. mosseae*. In addition, four and two genes for chalcone synthase and chalcone reductase, respectively, were found to be repressed. These three enzymes catalyze key reactions in the biosynthesis of phenylpropanoid compounds. Another series of duplicate experiments involving *G. margarita* supported this finding. Thus, the reproducibility of the repression of phenylpropanoid biosynthesis-related genes was confirmed unequivocally. Liu *et al.* presented a small list of AM-repressed genes.[16] Our finding that particular forms of phosphoenolpyruvate carboxylase and glutathione *S*-transferase are repressed is in accordance with their results. Hohnjec *et al.*,[18] Kistner *et al.*,[10] and Guimil *et al.*[41] presented larger lists of AM-repressed genes of *M. truncatula*, *L. japonicus*, and rice, respectively, but neither *PAL* genes nor chalcone synthase ones were included in the lists. In the work of Hohnjec *et al.*,[18] for example, many stress-related genes were listed as AM-repressed genes, because they were highly up-regulated in the phosphate-starved control roots. Very recently, Liu *et al.* presented the largest list of AM-repressed genes in *M. truncatula* roots as well as those in other portions.[20] Again, however, PAL genes were not included in their list of repressed genes. We will confirm our current results by promoter analysis and discuss the discrepancy (see below). Besides phenylpropanoid biosynthesis-related genes, a phosphate transporter gene (*LjPT1*) was also repressed (Table 2). This finding is in accord with the general tendency that the expression of common phosphate transporters is suppressed in AM roots.[44−46] A recently found AM root-enhanced phosphate transporter gene of *L. japonicus*[33] was not found on the present nylon filter.

3.3. Differential expression of plant genes caused by G. margarita infection in the initial stage of symbiosis

In contrast to the later stage of symbiosis (Table 1), a number of genes were found to be up-regulated or down-regulated on the initial association with the AM fungus (Table 3). In accordance with previous reports,[16,47−52] the genes for enzymes involved in defense-related secondary metabolism and the pathogen response, such as PALs, chalcone synthases, and peroxidases, were moderately up-regulated at this stage. A number of genes for transcription or translation were also induced, suggesting that a dynamic cellular change in plant roots occurs at the initial stage of the AM association. In addition, several genes involved in signal transduction were up-regulated (Table 3). For example, the gene for a pathogen-induced receptor protein kinase with a characteristic extracellular domain was induced.[53,54] Transcripts for a heterotrimeric G protein-coupled receptor, small GTP-binding proteins, protein serine/threonine kinases, and a mitogen-activated protein kinase were also accumulated. These gene products may represent signal transduction pathways for AM colonization.

Table 3. Transcriptional changes caused by *G. margarita* infection in the initial stage of symbiosis

Current annotation	Fold (Gi1/gni1)	Gene ID	gni1	Gi1	e-value
Amino acid and nitrogen metabolism					
Selenocysteine methyltransferase	2.24	MWM066h10_r	62	139	6E−25
Diaminopimelate decarboxylase	2.05	MWM149b05_r	128	262	1E−66
S-adenosyl methionine synthetase	2.02	MWM180f07_r	145	292	4E−67
VuP5CR	0.28	GENLf018g02	78	21	4E−24
Arginine decarboxylase	0.29	MWM198e12_r	102	29	2E−49
Aminotransferase 2	0.33	MWM222b09_r	101	33	5E−66
Proline dehydrogenase	0.36	MWM135h10_r	99	35	8E−29
Delta-1-pyrroline-5-carboxylate synthase	0.36	GENLf045e06	121	43	4E−09
Carbon metabolism					
Invertase	2.32	MWM224d02_r	150	347	6E−08
Sucrose synthase	2.27	MWL068h11_r	271	614	1E−58
Glucose-1-phosphate adenylyltransferase	2.13	MWM086h02_r	128	271	7E−65
Alpha-mannosidase	2.11	GENLf064h06	138	291	4E−24
Glucose-6-phosphate dehydrogenase	2.07	MR098a03_f	47	93	7E−06
Beta-amylase	2.06	GENf097b02	111	229	1E−132
Triosephosphate isomerase	2.05	MWM193g10_r	272	559	1E−77
Glucosyltransferase-like protein	2.01	MWL049f07_r	126	257	9E−06
Beta-D-xylosidase	0.26	MWM219c11_r	119	31	9E−55
Fructose-bisphosphate aldolase	0.29	MWM024h09_r	95	28	2E−39
Malonyl-CoA: acyl carrier protein transacylase	0.30	MWL014e01_r	115	34	4E−33
Putative 2-isopropylmalate synthase	0.31	GENf086f07	76	23	4E−34
Mannosyltransferase-like protein	0.32	MWM235d07_r	92	28	3E−04
Citrate synthase	0.37	MWM239b12_r	147	54	4E−38
Secondary metabolism					
Chalcone reductase	3.03	MWM002d07_r	98	306	1E−60
Phenylalanine ammonia-lyase (LjPAL2)	2.16	GENLf058e04	95	202	0.006
Phenylalanine ammonia-lyase (LjPAL1)	2.09	MRL007g11_f	136	283	9E−38
4-coumarate:CoA ligase	2.10	MWL020d04_r	110	235	3E−34
Chalcone synthase	2.05	MWL020g05_r	144	295	1E−14
Laccase	0.30	MWM219c08_r	124	36	3E−44
Putative diphenol oxidase	0.34	GENf055e07	87	29	1E−13
Transport/membrane					
Plasma membrane $Ca2^{+}$-ATPase	2.34	MWM178b05_r	169	396	3E−74
Aquaporin protein PIP1	2.34	MWL033d08_r	279	652	8E−51
Putative amino acid transporter	2.14	MWL064b03_r	71	150	8E−51
Putative ABC transporter protein	2.13	MWL077b08_r	158	335	1E−14
Putative nuclear transport factor	2.09	MWM105a05_r	118	248	1E−43
Vacuolar ATPase	2.06	MWM238d10_r	84	173	4E−56
Nuclear transport factor	2.02	GNf048c09	84	165	1E−38
Sorbitol transporter	2.01	MWM244a03_r	41	79	4E−57
MATE efflux family protein	0.23	GENLf013e12	111	25	2E−06
Cation-transporting ATPase	0.31	MWM087d06_r	154	46	3E−20
Plastidic phosphate translocator-like protein	0.32	GENf029g12	108	34	6E−96
Nodulin					
Early nodulin ENOD18	2.65	GENf079d10	34	90	2E−14

Continued

Table 3. Continued

Current annotation	Fold (Gi1/gni1)	Gene ID	gni1	Gi1	e-value
Signal transduction					
Protein phosphatase 2C	2.40	MWM035c07_r	248	596	1E−52
Protein serine/threonine kinase	2.35	MWM206a09_r	74	174	4E−29
G protein-coupled receptor	2.33	GENLf064g09	70	163	
Receptor protein kinase	2.31	MPDL044b10_f	56	130	7E−18
Small GTP-binding protein	2.18	MWM122f01_r	174	380	5E−44
GUN4 regulator	2.12	MPDL091h07_f	149	316	1E−63
Putative acid phosphatase	2.09	MWM048e06_r	415	866	8E−24
MAP kinase 3	2.09	MR062e03_f	582	1215	3E−84
Putative GTP-binding protein	2.05	MWM119e05_r	161	329	4E−34
Protein phosphatase-2C	2.05	MWM050f11_r	89	182	0.0003
Serine/threonine protein phosphatase	2.05	MR028a01_f	85	173	2E−27
Calcium-dependent protein kinase	2.01	MWM060b03_r	56	113	3E−05
Protein kinase	0.25	MWL036f06_r	128	32	3E−10
PAP-specific phosphatase	0.26	MWM204g03_r	104	28	7E−25
PP2A regulatory subunit	0.32	MWM123a10_r	124	39	4E−22
Hydrolase/ inositol or phosphatidylinositol phosphatase	0.37	MWM231g02_r	75	27	5E−27
Transcription/translation					
Putative bZIP transcription factor	3.10	MWM014e09_r	65	202	1E−30
Eukaryotic initiation factor	2.71	MWM099b12_r	380	1030	2E−04
Poly(A)-binding protein	2.40	MWM214d01_r	148	352	1E−44
Putative aspartate-tRNA ligase	2.34	MWM096c11_r	187	438	3E−23
Homeobox domain protein	2.07	MPD034c07_f	51	104	8E−79
Heat shock transcription factor	2.03	MR008f01_f	58	117	8E−39
Glycine-rich RNA-binding protein	2.01	GENLf028b01	189	379	3E−36
Putative squamosa promoter-binding protein	0.25	GENLf063g01	132	33	7E−07
SDL-1 plastid protein	0.29	GENLf045h01	112	32	9E−92
Transcription factor MYB4 homolog	0.31	MPD092a11_f	67	20	1E−22
Transcription regulatory protein	0.32	MWM065b02_r	116	37	
Putative DOF zinc finger protein	0.33	MWM178e06_r	118	38	1E−21
Putative translation initiation protein	0.33	GENLf057g11	106	32	
Cell wall					
Pectin acetylesterase	2.44	MWM096a11_r	174	424	9E−54
Putative pectinesterase	2.05	MWM097c10_r	278	570	3E−08
Pectinesterase	2.02	MWM132g12_r	85	172	1E−55
Callose synthase	0.36	GENLf063h05	92	32	2E−28
Protein fate					
Protein secretion pathway protein	2.08	GENLf046b01	124	257	
Dipeptidyl peptidase IV-like protein	0.27	MPDL020f10_f	106	28	7E−35
Oligopeptidase A	0.29	MWM031e10_r	131	37	3E−50
26S proteasome ATPase subunit	0.31	MWM223f06_r	80	24	3E−30
Putative ubiquitin carboxyl terminal hydrolase	0.33	MPDL041a06_f	108	35	2E−18
Pro-X carboxypeptidase-like protein	0.36	MPD016e03_f	86	31	
Serine protease inhibitor phloem serpin-1	0.37	GENf065b05	77	30	3E−26
Pathogen-related					
Respiratory burst oxidase protein D	2.57	GENLf020h11	54	138	3E−41

Continued

Table 3. Continued

Current annotation	Fold (Gi1/gni1)	Gene ID	gni1	Gi1	e-value
Syringolide-induced protein	2.43	MWM033e05_r	123	296	8E−14
Peroxidase 3 precursor	2.12	MWM241c09_r	42	88	1E−13
Syringolide-induced protein 14-1-1	2.06	MWM031c04_r	95	195	8E−27
Disease resistance-related protein	2.04	MWM067e07_r	74	153	6E−23
Class III peroxidase PSYP1	2.03	MWL018a05_r	82	166	2E−37
Endo-1,4-beta-glucanase	0.14	MWL011b05_r	244	34	9E−30
Syringolide-induced protein	0.35	MWM037b07_r	139	48	2E−07
Syringolide-induced protein	0.41	MWM014d11_r	102	41	8E−64
Phytohormone-related					
Auxin-repressed protein	2.13	MPDL064h08_f	270	574	1E−14
Cytokinin oxidase	0.24	MWM042d03_r	129	28	3E−35
Other enzymes					
Cytochrome P450	2.52	MWM049d04_r	234	589	8E−55
Cytochrome P450	2.51	MR061f02_f	167	418	7E−35
Phosphogluconate dehydrogenase	2.47	MWM228b11_r	193	475	1E−49
Fatty acid hydroxylase cytochrome P450	2.28	MWM051a05_r	88	201	3E−26
Cytochrome P450	2.21	MR043g06_f	465	1027	2E−09
Cytochrome P450	2.20	MWM152a11_r	43	93	4E−29
Epoxide hydrolase	2.11	MWM079e11_r	56	119	4E−54
Thiazole biosynthetic enzyme	2.08	MWM107g04_r	150	311	7E−33
Putative helicase	2.03	MWL079f07_r	157	317	7E−11
Phosphatidylserine decarboxylase	2.02	MWM214c03_r	65	131	1E−57
Isopentenyl-diphosphate isomerase II	0.21	MWM082f11_r	123	25	2E−26
Retroelement pol polyprotein-like	0.24	MWL062c10_r	129	30	2E−26
Histone acetyltransferase HAT B	0.24	MWM193c03_r	89	20	4E−13
Thiamine biosynthetic enzyme	0.25	GENf012a12	102	25	3E−57
Cytochrome P450	0.32	MWM170d07_r	158	50	2E−63
Obtusifoliol 14-alpha demethylase	0.33	GENf014g11	137	45	5E−54
Magnesium chelatase	0.34	MWL046f07_r	81	27	5E−56
UMP synthase	0.35	MWM187d07_r	120	41	3E−53
Putative cytochrome P450	0.35	MWM139c03_r	95	33	3E−59
Dihydroneopterin aldolase	0.36	GENLf038a07	74	26	2E−24
Other categories					
Polyubiquitin 4	2.60	MWM214g11_r	119	308	3E−09
Metallothionein-like protein class II	2.54	MWM200f03_r	1109	2821	3E−19
CPRD49	2.42	MWM128g09_r	148	357	1E−17
Ubiquitin precursor	2.28	MWM011f03_r	544	1242	2E−10
DnaJ-like protein	2.04	MWM184b12_r	141	288	8E−71
Bax inhibitor-1 like	2.04	MWM016c06_r	143	293	4E−50
Heat shock protein 70 cognate	2.02	MWM159a01_r	174	351	2E−62
Putative 2Fe-2S iron–sulfur cluster protein	2.01	MPD065e04_f	51	102	3E−07
Ankyrin-repeat protein	2.00	MWM067b10_r	428	856	8E−17
Vacuolar sorting receptor protein BP-80	2.00	MWL009b09_r	173	345	9E−20
Calcineurin B-like protein	0.25	MWM143g03_r	82	20	0.007
Peroxiredoxin Q	0.31	MWM126d02_r	97	29	1E−16

Continued

Table 3. Continued

Current annotation	Fold (Gi1/gni1)	Gene ID	gni1	Gi1	e-value
Senescence-associated putative protein	0.33	MWL051e09_r	110	35	1E−16
PSII low MW protein	0.34	MWL078e10_r	578	198	4E−19
Histone H2A	0.35	MWM209e08_r	133	46	9E−51
Actin	0.36	GENf007a10	82	29	1E−48
Phosphatidylinositol transfer-like protein IV	0.36	GENf020a06	125	44	6E−20
Chlorophyll a/b-binding protein type II	0.36	MPD059g06_f	95	34	7E−46
Early light-inducible protein	0.37	MWL040f08_r	85	31	2E−43
Cytochrome b/f	0.37	MWM225h10_r	325	120	4E−71

Because there is a varying lag time between sporulation and the arrival of AM hyphae on the host roots, the initial response to mycorrhizae is not necessarily synchronous. Therefore, we modified the 'nurse pot' method[30] as described under Materials and Methods. Freshly prepared sterile seedlings were transplanted into containers containing *L. japonicus* plants well-colonized by *G. margarita* or mock-inoculated plants and then allowed to grow for a week. Then, roots of the younger seedlings were harvested from the container inoculated with *G. margarita* (Gi1) or the mock-inoculated container (gni1). Radio-labeled target cDNAs were synthesized from total RNAs in the roots and then hybridized to a nylon filter cDNA array.[26] The normalized expression levels are shown in the gni1 and Gi1 columns. The expression levels relative to the mock-infected controls are given in the fold column. Genes that match hypothetical proteins of unknown function and ones that encode proteins exhibiting no homology to thus far known ones have been omitted from this table.

3.4. Expression patterns of PAL genes in L. japonicus

PALs connect primary and secondary metabolism in plants, catalyzing common rate-limiting steps of flavonoid phytoalexin synthesis, lignin synthesis, salicylic acid synthesis, etc. The expression patterns of PAL genes in our experiments are very characteristic compared with those in previous studies.[10,12−20,41] Since PAL genes are known to form a family in a number of plant species,[55] we first checked how many PAL genes were present on the array membrane and found nine non-redundant ones. In addition, we found a TAC clone (Accession no. AP004502) containing a unique PAL gene, *LjPAL5*, in the databases. As shown in Table 4, most PAL genes were induced in the initial stage of AM infection and then repressed in the later stage. However, *LjPAL10* did not seem to be expressed differentially. In addition, other genes, *LjPAL7 and LjPAL9*, might be of the intermediate type. Thus, as pointed out previously,[55] care must be taken that PAL genes do not show similar expression patterns. Although the array membrane was washed under high-stringency conditions after hybridization, cross hybridization among the gene family members could not be excluded since the members are more than 80% identical to each other at the nucleotide level in

Table 4. *L. japonicus* genes for PALs and their expression patterns

Gene name	Gene ID	Gi1/gni1	Gm6/SC3	Gm6/SC3 (RT−PCR)	Gi8/gni3
LjPAL1	MRL007g11_f	2.08 (283/136)	0.24 (107/445)	**0.35 ± 0.17**	0.17 (80/466)
LjPAL2	GENLf058e04	2.20 (202/92)	0.26 (72/274)	**0.29 ± 0.11**	0.22 (84/384)
LjPAL3	MR060a09_f	1.70 (426/250)	0.15 (148/999)	**0.23 ± 0.16**	0.17 (186/1093)
LjPAL4	GENLf025c04	1.49 (202/136)	0.16 (69/431)	**0.09 ± 0.09**	0.17 (80/458)
LjPAL5				**0.16 ± 0.08**	
LjPAL6	MWL047f06_r	1.53 (197/129)	0.56 (160/286)	**0.10 ± 0.03**	0.33 (114/344)
LjPAL7	MWL052f09_r	1.54 (330/214)	0.77 (312/407)	**0.40 ± 0.27**	0.22 (190/873)
LjPAL8	MWL032c01_r	0.93 (165/178)	0.25 (67/273)	**0.09 ± 0.04**	0.26 (96/361)
LjPAL9	MWM088g05_r	1.33 (326/245)	0.95 (621/652)	**0.26 ± 0.08**	0.34 (207/612)
LjPAL10	MR078c05_f	1.44 (340/236)	1.15 (161/140)	**1.34 ± 0.56**	0.67 (166/248)
ß-actin				**1.35 ± 1.38**	

The lightface data in the Gi1/gni1, Gi8/gni3 and Gm6/SC3 columns are the fold values for gene expression in roots at 1 week and 8 weeks after inoculation of *G. margarita*, and 6 weeks after inoculation of *G. mosseae*, respectively, compared with those for control roots. The normalized expression levels observed in the array analyses are also given in parentheses. The boldface Gm6/SC3 column shows the results of real time RT−PCR (mean ± SD for four replicates) for gene expression in roots at 6 weeks after inoculation of *G. mosseae*, compared with those for control roots.

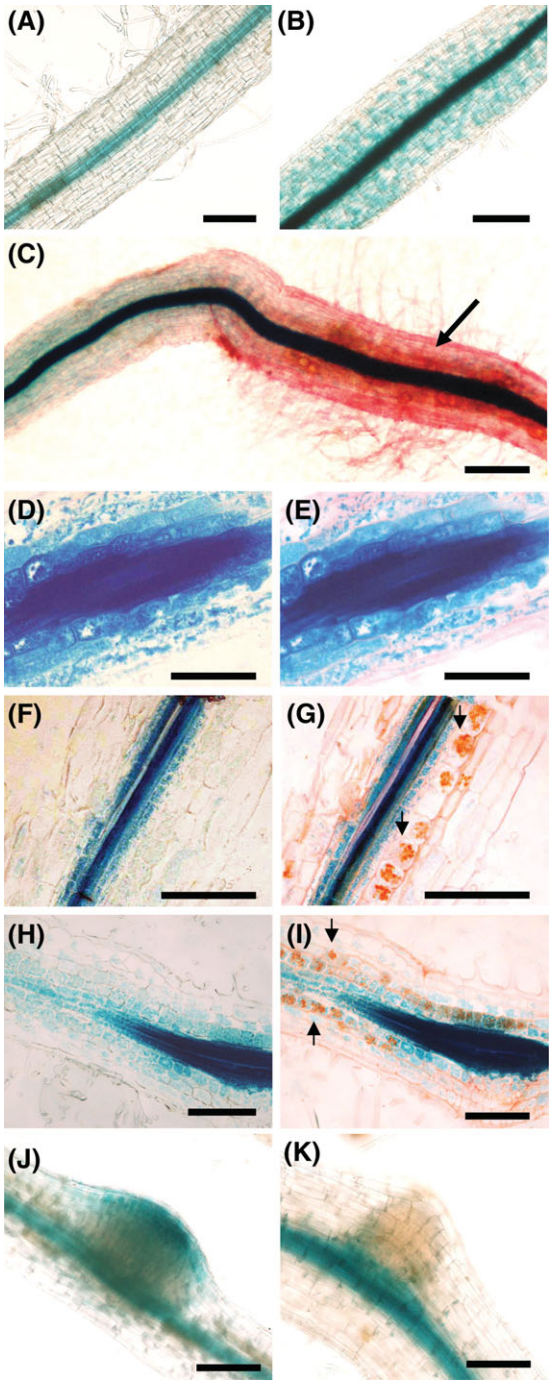

Figure 2. Spatial patterns of *LjPAL1* promoter activity in *L. japonicus* hairy roots. GUS activity is visible as a blue color in intact roots (**A**–**C**) or longitudinal sections of AM roots (**D**–**I**). A, a non-infected root. (**B**), 4 weeks after inoculation of the sieved carrier containing contaminating microorganisms. (**C**) An AM root, 4 weeks after addition of the whole *G. mosseae* inoculum. The arrow indicates the region heavily colonized by the AM fungus. After regular GUS staining, the root was re-stained with safranin. Sections of GUS-stained roots were prepared (**D**, **F**, and **H**) and then stained on slide glasses with safranin (**E**, **G**, and **I**). The small arrows in G and I indicate arbuscules of the AM fungus. (**J** and **K**) *LjPAL1* promoter activity in nodules on *L. japonicus* hairy roots. Two weeks after inoculation of *M. loti*, GUS activity in the nodulated roots was examined under a stereomicroscope. (**J**) A nodule primordium. (**K**) A more mature nodule. Bars, 100 μm.

their coding regions. Therefore, we performed real-time RT–PCR experiments with gene-specific primer sets to validate the differential expression of the PAL genes. The results of RT–PCR for all PAL genes were more or less the same as those of array analysis (Table 4). In addition, we found that the *LjPAL5* gene, which was not found on the array membrane, was severely down-regulated in AM roots (Table 4).

To further confirm the repression of some PAL genes after AM colonization, we searched for genomic sequences of the PAL genes in databases, finding that *LjPAL1*, *LjPAL4*, and *LjPAL5* lie in tandem on a single TAC clone, AP004502. We chose the *LjPAL1* promoter, which shows typical differential expression, for further analysis. This promoter, 2 kb in size, was amplified by PCR, fused with the *uidA* reporter for GUS, and then introduced into *L. japonicus* by the hairy root method with *A. rhizogenes*. The transformants showed basal activity, especially in central cylinders, in the absence of any microorganisms (Fig. 2A). The GUS activity was augmented throughout the roots in the presence of contaminating microorganisms in the sieved carrier (Fig. 2B). When the transformants were inoculated with the whole *G. mosseae* inoculum, the area of expression decreased (Fig. 2C). The specific GUS activity levels in the entire hairy roots of the above transformants were 1.2 ± 0.2, 7.1 ± 2.9, and 4.1 ± 1.5 pmol/min/μg protein, respectively. Unexpectedly, when GUS-stained AM roots were re-stained with safranin, a red dye that stains fungal cells better than plant cells, it turned out that *G. mosseae* only colonized where GUS activity was low (Fig. 2C). To confirm this observation, sections of GUS-stained AM roots were prepared and then re-stained with safranin. As shown in Fig. 2D and E, the root portions exhibiting high *LjPAL1* promoter activity did not contain *G. mosseae*. In contrast, the AM fungus colonized well where the GUS level was low (Fig. 2F and G). In some cases, *G. mosseae* was observed where GUS activity was also significant, but the level of GUS was not very high either (Fig. 2H and I). As described above, the whole *G. mosseae* inoculum and the sieved carrier contained equivalent amounts of contaminating microorganisms. Therefore, host plants repress PAL gene expression where AM fungi colonize, preventing infection by pathogenic microorganisms. This repression pattern is similar to that of isoflavone reductase of *M. truncatula* previously reported,[50] but different from those of PAL and chalcone synthase observed in that study. Comprehensive expression analysis of every family member for the latter enzymes of *M. truncatula* would be necessary to resolve this discrepancy.

3.5. Commonly repressed genes of L. japonicus in AM roots and nitrogen-fixing nodules

When the results of cDNA array experiments on AM roots with *G. mosseae*, and ones on *G. margarita* and

Table 5. Co-regulated genes of *L. japonicus* in AM roots and nitrogen-fixing nodules

Current annotation	Gene ID	Gm6/SC3	Nod
Beta-amylase-like protein	MWL048f05_r	4.6	2.9
Chitinase	MWM140d02_r	4.3	6.6
Chalcone reductase	GNf040a09	4.1	5.6
Asparagine synthetase	MWL032c11_r	4.0	67.2
Asparagine synthetase	GNf053e06	4.0	34.2
Chitinase	MWM034g12_r	3.9	13.4
Asparagine synthetase	MWM233f05_r	3.3	25.1
Putative PGPD14 protein	MWL059c01_r	2.7	3.9
Sterigmatocystin biosynthesis protein	GNf018c04	2.6	3.4
Branched chain alpha-keto acid dehydrogenase	MWM092a07_r	2.6	5.0
Seed imbibition protein, putative	MWL069f08_r	2.5	3.4
Annexin	MPD065b05_f	2.4	4.4
Asparagine synthetase	GNf021f11	2.4	30.2
Nodule-enhanced sucrose synthase	MWL080e04_r	2.3	4.8
Annexin	MPD042e01_f	2.2	6.1
Phenylalanine ammonia-lyase (LjPAL5)	MWM056d02_r	0.13	0.12
Phenylalanine ammonia lyase (LjPAL3)	MR060a09_f	0.15	0.14
Phenylalanine ammonia-lyase (LjPAL4)	GENLf025c04	0.16	0.11
Histidine decarboxylase	GENf054a02	0.17	0.05
Naphthalene dioxygenase iron sulfur protein	MPDL068f03_f	0.17	0.16
Cytochrome P450-1	MR095g09_f	0.17	0.06
Phosphate transporter	MWM077d10_r	0.18	0.25
Transcription factor WRKY4	MWM168c07_r	0.19	0.15
Lupeol synthase	GNf046g09	0.19	0.30
ATP synthase 9	MWM223c10_r	0.20	0.14
NAD(P)H dependent 6′-deoxychalcone synthase	MWM174f04_r	0.21	0.31
Similar to the BURP domain	MPDL062c05_f	0.22	0.13
HSP100/ClpB	MRL022b06_f	0.23	0.15
Syringolide-induced protein B13-1-9	MWM033e05_r	0.23	0.14
Histidine decarboxylase, putative	MWM231b10_r	0.24	0.04
Phenylalanine ammonia-lyase (LjPAL1)	MRL007g11_f	0.24	0.17
Glycogen synthase kinase-3 homolog MsK-3	MWL017b06_r	0.24	0.31
Phenylalanine ammonia-lyase (LjPAL8)	MWL032c01_r	0.25	0.19
Seed coat BURP domain protein	MPDL082d06_f	0.25	0.18
Ribonuclease non-S	MWM082g02_r	0.26	0.31
Putative zinc finger POZ protein	MWM026d08_r	0.26	0.09
WRKY transcription factor	GENLf072f04	0.26	0.30
Phenylalanine ammonia-lyase (LjPAL2)	GENLf058e04	0.26	0.17
Phosphate transporter	MR054e04_f	0.27	0.27
Chalcone synthase	MWM170f10_r	0.27	0.30
WRKY transcription factor	MWM029g02_r	0.28	0.17
Cytochrome P450	MWL061f11_r	0.28	0.05
Syringolide-induced protein 14-1-1	GNf002b04	0.29	0.20
Cytochrome P450, putative	MR076b02_f	0.30	0.06
Putative anthocyanidine rhamnosyl-transferase	GNf060a01	0.30	0.30

The gene expression levels in roots 6 weeks after inoculation with the whole inoculum of *G. mosseae* relative to those with the sieved carrier (Gm6/SC3) and those in mature nodules (4 weeks after inoculation of *M. loti*) relative to in non-infected roots (Nod) were compared. Genes of which the expression levels relative to controls were >2.2 or <0.30 are listed up. Defense-related or stress-induced genes are highlighted in bold. Genes that match hypothetical proteins of unknown function and ones that encode proteins with no homology to thus far known ones have been omitted from this table.

mature root nodules with *M. loti* were compared with each other, the overlapping of induced genes or repressed genes was found to be limited (Supplementary Fig. S2), in accord with previous reports.[17,18] However, when the commonly regulated genes in *G. mosseae*-colonized roots and mature root nodules were listed up, it was obvious that many defense-related and stress-induced genes were included in the commonly repressed list (Table 3). They include genes for WRKY transcription factors, which are up-regulated in response to biotic or abiotic stress,[40,56] and those for BURP domain proteins, one of which is a stress-induced transcription factor,[57] besides PAL genes. These results suggest that host plants accept AM fungi and compatible rhizobia in similar manners, their defense mechanisms being suppressed.

Because *LjPAL1* is one of the commonly repressed genes in AM roots and nodules (Table 5), we inoculated *M. loti* into hairy roots transformed with the *LjPAL1* promoter-GUS construct. As shown in Fig. 2J and K, strong GUS activity was detected at the top of a nodule primordium, but it had soon disappeared in a slightly more mature nodule, in accordance with the results of the array experiments (Table 5).

3.6. Concluding remarks

We performed comprehensive transcriptome analysis and spatial examination of gene expression in AM roots and root nodules of *L. japonicus*, taking into account the effects of contaminating microorganisms. We found that several cysteine protease genes were specifically induced in arbuscule-containing cells of AM roots. Moreover, we also found that PAL and other phenylpropanoid biosynthesis-related genes were moderately induced on the initial infection of the symbionts and then repressed concomitant with the establishment of the two symbioses. Characteristic expression patterns were observed both in the absence of contaminating microorganisms (Table 4, experiments with *G. margarita*; Fig. 2J and K) and more drastically in their presence (Table 4, experiments with *G. mosseae*; Fig. 2A–I). So far, it has been suggested that defense genes for AM fungi or rhizobia are initially up-regulated and then down-regulated.[16,26,47–52,58] Nevertheless, the current study is unexpectedly the first demonstration that this prediction is correct especially for AM root formation with *G. mosseae* and *G. margarita* using a large scale cDNA array. Then, why did previous works on AM roots not reveal the unique expression patterns of PAL and other phenylpropanoid biosynthesis-related genes? When the expression levels of these genes in roots with commercial inoculants of AM fungi applied were examined,[12–15,17–19] it is possible that their induction by contaminating microorganisms and their repression by AM fungus colonization were super-imposed, resulting in comparable levels to those in sterile non-infected roots. Actually, when we did a similar experiment,[12–15,17–19] we did not detect the differential expression of most PAL genes except *LjPAL10*, which was moderately up-regulated (Supplementary Table S1). Other previous works in which aseptic spores of AM fungi were inoculated did not show significant down-regulation of these phenylpropanoid biosynthesis-related genes, either.[16,20,41] On the other hand, our experiments involving NaClO-treated *G. margarita* spores revealed repression of the genes. It is difficult at present to fully explain this discrepancy. As revealed in this work, however, the varying microbial population around AM roots significantly affects gene expression and hence the reproducibility of the experiments. If our surface-sterilization of the spores was not complete, for example, the differential expression of plant genes on *G. margarita* colonization might be similar to that on application of a commercial *G. mosseae* inoculant.

The presence of contaminating microorganisms is, in a sense, closer to natural field conditions than the inoculation of aceptical spores of AM fungi into sterile plants. The spatial investigation in this study revealed that a PAL gene, *LjPAL1*, is repressed where AM fungi colonized. Although PALs are multifunctional enzymes, we consider that the defense response including *de novo* synthesis of flavonoid phytoalexins against other microorganisms than AM fungi is suppressed. In nature, host plants may accept microsymbionts by suppressing their defense reactions to a minimum level at which they may still prevent infection by pathogens.

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