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

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(Received on 17 January 2007; accepted on June 22, 2007)

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* 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.1,2 Additionally, AM fungi endow plants with tolerance to pathogens and abiotic stress.3,4 In the process of colonization by AM fungi, the hyphae of extra-radical 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...
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. 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.

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.

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, the subtractive hybridization approach, and cDNA and oligonucleotide array analyses have been performed for *Medicago truncatula*, a model legume. *Medicago truncatula* was also used to investigate the differential expression of chitinase genes in AM colonization, nodulation, and plant–pathogen interactions. *Lotus japonicus* is another valuable model legume. For example, *L. japonicus* has been used for elucidation of the molecular mechanisms of plant–AM fungi interactions. Gene expression profiling with the aid of cDNA-amplified fragment length polymorphism has also been carried out.

For transcriptome analyses of host responses to AM fungi, we here made use of a large-scale cDNA array of *L. japonicus*, 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 μE m⁻² s⁻¹ 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 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 after staining with trypan blue.

When the initial stage of AM symbiosis was examined, we modified the ‘nurse pot’ method, 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. 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,
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.31,33 The forward and reverse primer sets and annealing temperatures (in parentheses) were as follows: 5′-CAGTGACAAAAGGTGTGGAGCTAC-3′ and 5′-GTCCAGGGTGGTGCTTAAGCC-3′ for LjPAL1; 5′-GCTCAGGTGCTGCCAGTTATGTT-3′ and 5′-GACCTTGTGTTTTTCTGTAGTG-3′ for LjPAL2; 5′-AATTGATCACTTAGT-3′ and 5′-AAGAACAGTTTGTTTGTTTGAG-3′ for LjPAL3; 5′-CCCCATGTTGTTTTTCTGTAGTG-3′ and 5′-AAGAACAGTTTGTTTGTTTGAG-3′ for LjPAL4; 5′-AGAACAGTTTGTTTGTTTGAG-3′ and 5′-CTCTTGTGTTTTTCTGTAGTG-3′ for LjPAL5; 5′-AGAACAGTTTGTTTGTTTGAG-3′ and 5′-GCTCAGGTGGCTGCCATCGCC-3′ for LjPAL6; 5′-GCTCAGGTGGCTGCCATCGCC-3′ and 5′-GCTCAGGTGGCTGCCATCGCC-3′ for LjPAL7; 5′-GCTCAGGTGGCTGCCATCGCC-3′ and 5′-GCTCAGGTGGCTGCCATCGCC-3′ for LjPAL8; 5′-GCTCAGGTGGCTGCCATCGCC-3′ and 5′-GCTCAGGTGGCTGCCATCGCC-3′ for LjPAL9; and 5′-GCTCAGGTGGCTGCCATCGCC-3′ and 5′-GCTCAGGTGGCTGCCATCGCC-3′ 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 BamHI site. Therefore, to amplify the region derived from genomic DNA of L. japonicus, forward primer 5′-ATGCGGCGCTGACCACATGGTTATGAAC TAGCC-3′ and reverse primer 5′-ATTGATCATTTAGTATATGATCTCTCATTACA-3′, containing NosI and BclI sites, respectively, were used for PCR. The BclI end of the promoter was ligated to the BamHI 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 SalI sites at the ends of the intermediate construct, the promoter-GUS unit was ligated into the SalI site of pHKN29, 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 Schlamann, Leiden University, as described previously.33,35 Transformsants with green fluorescent protein (GFP)-positive hairy roots were transferred to vermiculite containing the modified Hornum solution, inoculated with the entire G. mossae incolum or sieved carrier, and then grown as described above. When nodule formation was examined, the transformsants 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-glucuronicid, 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 (Herus 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, respectively. It has been pointed out that fungal transcripts account for up to 12% of the entire transcripts of AM roots, 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, 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      | GN055c06       | 1005   | 3E−49   |
| Asparagine synthetase                                   | 0.9     | 3.3     | 1.8      | MWM233d05_r    | 460    | 3E−36   |
| Asparagine synthetase                                   | 1.1     | 2.4     | 1.4      | GN021f11       | 569    | 4E−81   |
| **Carbon metabolism**                                   |         |         |          |                |        |         |
| Alpha-mannosidase                                       | 1.4     | 3.6     | 2.7      | MPDL053b07_f   | 217    |         |
| Alpha-mannosidase                                       | 1.3     | 3.2     | 1.8      | MPDL018c02_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      | GN040a09       | 231    | 6E−06   |
| Chalcone reductase                                       | 0.7     | 2.1     | 2.3      | GN086d03       | 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      | MWM070a05_r    | 1440   | 9E−56   |
| Putative nitrate transporter                             | 0.9     | 2.2     | 2.6      | MWM134b03_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      | MWM091d06_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      | MWM080e04_r    | 797    | 2E−78   |
| **Signal transduction**                                 |         |         |          |                |        |         |
| Annexin                                                 | 1.2     | 3.2     | 2.8      | MPD097c02_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      | MWM231b03_r    | 24488  | 4E−39   |
| **Protein fate**                                        |         |         |          |                |        |         |
| Cysteine proteinase (LjCyp4)                            | 3.8     | 19.7    | 8.0      | GN089d01       | 664    | 6E−40   |
| Cysteine proteinase (LjCyp1)                            | 1.9     | 10.6    | 3.3      | GN032f12       | 251    |         |
| Cysteine proteinase (LjCyp2)                            | 1.7     | 5.0     | 2.3      | GN037h07       | 964    | 6E−23   |
| Cysteine proteinase (LjCyp3)                            | 1.3     | 4.0     | 2.3      | GN071h01       | 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      | MPDL019c09_f   | 980    | 5E−15   |
| **Phytohormone-related**                               |         |         |          |                |        |         |
| Jasmonic acid 2                                         | 1.4     | 6.0     | 4.5      | MWM076b07_r    | 149    | 7E−73   |
| **Other enzyme**                                        |         |         |          |                |        |         |
| Nicotianamine synthase                                  | 4.7     | 5.3     | 2.1      | GN070f09       | 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, 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.
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. Annexin genes were also induced in AM roots, in accordance with Manthey et al. Four cysteine proteinase genes, designated as LjCyp1-4, were most obviously up-regulated among the AM-enhanced genes (Table 1), confirming previous reports. 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, serine carboxypeptidase, annexin, and calcium-binding protein genes were reported to be expressed not only in arbuscule-containing cells but also in the cells around them. In contrast, the endoglucanase (MtCel1), cysteine-rich antifungal protein, and AM-induced phosphate transporter 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, 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 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.

A promoter region of a calcium-binding protein gene of L. japonicus was reported to be activated during AM development. 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...
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/gmi3 | 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 | MWM088403_r | 297 | 5E−68 |
| UDP-glucose protein transglycosylase | 0.49 | 0.32 | 0.54 | MWM177b05_r | 429 | 3E−32 |
| Glucose-6-phosphate 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 | GENL025e04 | 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 | MWL032b01_r | 430 | 2E−40 |
| Phenylalanine ammonia-lyase (LjPAL2) | 0.27 | 0.26 | 0.22 | GENL008s04 | 411 | 0.006 |
| Chalcone synthase | 0.44 | 0.27 | 0.39 | MWM170f10_r | 536 | 8E−48 |
| Chalcone synthase | 0.28 | 0.36 | 0.63 | MWM193b03_r | 714 | 3E−80 |
| Chalcone synthase | 0.50 | 0.39 | 0.65 | MWL020g05_r | 450 | 1E−14 |
| Deoxylchalcone synthase | 0.40 | 0.21 | 0.36 | MWM174f04_r | 485 | 1E−18 |
| Chalcone reductase | 0.28 | 0.19 | 0.17 | MWM020d07_r | 709 | 1E−60 |
| Chalcone reductase | 0.35 | 0.22 | 0.17 | GN090d05 | 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 | GN046g09 | 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 Ca²⁺-ATPase | 1.05 | 0.38 | 0.26 | GENL026c07 | 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 | MWM048s01_r | 1670 | 8E−24 |
| **Transcription/translation** | | | | | | |
| Transcription factor WRKY4 | 0.35 | 0.19 | 0.18 | MWM168s07_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 | GN069g02 | 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 | GN002404 | 258 | 2E−23 |
| Syringolide-induced protein | 0.99 | 0.39 | 0.15 | GN095h04 | 258 | 6E−31 |
| Similar to the BURP domain | 0.56 | 0.22 | 0.13 | MPDL062e05_f | 4760 | 1E−48 |
| Seed coat BURP domain protein | 0.42 | 0.25 | 0.15 | MPDL082d06_f | 3753 | 9E−59 |

Continued
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*34 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

| Amino acid and nitrogen metabolism | Fold (Gi1/gni1) | Gene ID | gi1  | Gi1  | e-value |
|------------------------------------|-----------------|---------|------|------|---------|
| Selenocysteine methyltransferase    | 2.24            | MWM066h10_r | 62  | 139  | 6E−25   |
| Diaminopimelate decarboxylase      | 2.05            | MWM149b05_r | 128 | 262  | 1E−66   |
| 3-adenosyl methionine synthetase   | 2.02            | MWM180f07_r | 145 | 292  | 4E−67   |
| VuP5CR                            | 0.28            | GENL018g02 | 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   | GENL045e06 | 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 adenyltransferase | 2.13        | MWM086h02_r | 128 | 271  | 7E−65   |
| Alpha-mannosidase                  | 2.11            | GENL064b06 | 138 | 291  | 4E−24   |
| Glucose-6-phosphate dehydrogenase  | 2.07            | MR098s03_f | 47  | 93   | 7E−06   |
| Beta-amylase                       | 2.06            | GEN097t02 | 111 | 229  | 1E−132  |
| Triosephosphate isomerase          | 2.05            | MWM193g10_r | 272 | 559  | 1E−77   |
| Glucosyltransferase-like protein   | 2.01            | MWL049d07_r | 126 | 257  | 9E−06   |
| Beta-n-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          | GEN086d07 | 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            | MWM062d07_r | 98  | 306  | 1E−60   |
| Phenylalanine ammonia-lyase (LjPAL2) | 2.16          | GENL055e04 | 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            | GEN055e07 | 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            | GEN048d09 | 84  | 165  | 1E−38   |
| Sorbitol transporter                | 2.01            | MWM244a03_r | 41  | 79   | 4E−57   |
| MATE efflux family protein          | 0.23            | GENL013c12 | 111 | 25   | 2E−06   |
| Cation-transporting ATPase          | 0.31            | MWM087d06_r | 154 | 46   | 3E−20   |
| Plastidic phosphate translocator-like protein | 0.32   | GEN029g12 | 108 | 34   | 6E−96   |

*No. 3*] Y. Deguchi et al. 125
| Current annotation                        | Fold (Gi1/gn1) | Gene ID    | gi1   | 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           | GENL064g09 | 70    | 163 |         |
| Receptor protein kinase                  | 2.31           | MPDL044b10_f| 56    | 130 | 7E−18   |
| Small GTP-binding protein                | 2.18           | MWM12201_r | 174   | 380 | 5E−44   |
| GUN4 regulator                           | 2.12           | MPDL091h07_f| 149   | 316 | 1E−63   |
| Putative acid phosphatase                | 2.09           | MWM048c06_r| 415   | 866 | 8E−24   |
| MAP kinase 3                             | 2.09           | MR062e03_f | 582   | 1215| 3E−84   |
| Putative GTP-binding protein             | 2.05           | MWM119c05_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           | MWM014c09_r| 65    | 202 | 1E−30   |
| Enkaryotic 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           | MPDL034c07_f| 51    | 104 | 8E−79   |
| Heat shock transcription factor          | 2.03           | MR008f01_f | 58    | 117 | 8E−39   |
| Glycine-rich RNA-binding protein         | 2.01           | GENL028f01 | 189   | 379 | 3E−36   |
| Putative squamosa promoter-binding protein| 0.25           | GENL063g01 | 132   | 33  | 7E−07   |
| SDL-1 plastid protein                    | 0.29           | GENL045h01 | 112   | 32  | 9E−92   |
| Transcription factor MYB4 homolog        | 0.31           | MPDL092a11_f| 67    | 20  | 1E−22   |
| Transcription regulatory protein         | 0.32           | MWM065b02_r| 116   | 37  |         |
| Putative DOF zinc finger protein         | 0.33           | MWM178s06_r| 118   | 38  | 1E−21   |
| Putative translation initiation protein  | 0.33           | GENL057g11 | 106   | 32  |         |
| **Cell wall**                            |                |            |       |     |         |
| Pectin acetylglucosyltransferase         | 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           | GENL063h05 | 92    | 32  | 2E−28   |
| **Protein fate**                         |                |            |       |     |         |
| Protein secretion pathway protein        | 2.08           | GENL046b01 | 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           | MPDL016c03_f| 86    | 31  |         |
| Serine protease inhibitor phloem serpin-1| 0.37           | GENL065f05 | 77    | 30  | 3E−26   |
| **Pathogen-related**                     |                |            |       |     |         |
| Respiratory burst oxidase protein D      | 2.57           | GENL020h11 | 54    | 138 | 3E−41   |
| Current annotation                                | Fold (G1/gien) | Gene ID  | gien  | G1    | 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   |
| **Phytokrhome-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          | MR061h02_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          | MWL079b07_r | 157   | 317   | 7E−11   |
| Phosphatidylserine decarboxylase                | 2.02          | MWM214c03_r | 65    | 131   | 1E−57   |
| Isopenetyl-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          | GEN012a12   | 102   | 25    | 3E−57   |
| Cytochrome P450                                  | 0.32          | MWM170d07_r | 158   | 50    | 2E−63   |
| Obtusifoliol 14-alpha demethylase                | 0.33          | GEN014g11   | 137   | 45    | 5E−54   |
| Magnesium chelatase                             | 0.34          | MWL046d07_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          | GEN038a07   | 74    | 26    | 2E−24   |
| **Other categories**                            |               |           |       |       |         |
| Polyubiquitin 4                                  | 2.60          | MWM214g11_r | 119   | 308   | 3E−09   |
| Metallothionein-like protein class II           | 2.54          | MWM209f03_r | 1109  | 2821  | 3E−19   |
| CFRD49                                           | 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−11   |
| 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 (GI/gn1) | Gene ID   | gn1  | G1  | 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          | MW209968_r | 133  | 46  | 9E-51   |
| Actin                                                    | 0.36          | GNE907a10  | 82   | 29  | 1E-48   |
| Phosphatidylinositol transfer-like protein IV            | 0.36          | GNE820a06  | 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          | MWL04080_r | 85   | 31  | 2E-43   |
| Cytochrome b/f                                           | 0.37          | MW225k10_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 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. 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. Since PAL genes are known to form a family in a number of plant species, 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, 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/gn1 | Gm6/SC3 | Gm6/SC3 (RT-PCR) | GI8/gn3 |
|-----------|---------|---------|---------|-----------------|--------|
| LjPAL1    | MRL007g11_f | 2.08 (283/136) | 0.24 (107/445) | 0.35 ± 0.17 | 0.17 (80/466) |
| LjPAL2    | GENL058c04 | 2.20 (202/94)  | 0.26 (72/274)  | 0.29 ± 0.11 | 0.22 (84/384) |
| LjPAL3    | MR060h09_f | 1.70 (426/250) | 0.15 (148/999) | 0.23 ± 0.16 | 0.17 (186/1093) |
| LjPAL4    | GENL025c04 | 1.49 (202/136) | 0.16 (69/431)  | 0.09 ± 0.09 | 0.17 (80/458) |
| LjPAL5    |          |          |         | 0.16 ± 0.08   |        |
| LjPAL6    | MRL047806_r | 1.53 (197/129) | 0.56 (160/286) | 0.10 ± 0.03 | 0.33 (114/344) |
| LjPAL7    | MRL052809_r | 1.54 (339/214) | 0.77 (312/407) | 0.40 ± 0.27 | 0.22 (190/873) |
| LjPAL8    | MRL032e01_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) |
| β-actin   |          |          |         | 1.35 ± 1.38   |        |

The lightface data in the GI1/gn1, GI8/gn3 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.
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 $1.2 \pm 0.2$, $7.1 \pm 2.9$, and $4.1 \pm 1.5$ 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...
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. 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, and those for BURP domain proteins, one of which is a stress-induced transcription factor, 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).

**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 | GN040a09 | 4.1 | 5.6 |
| Asparagine synthetase | MWL032c11_r | 4.0 | 67.2 |
| Asparagine synthetase | GN053e06 | 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 | GN018c04 | 2.6 | 3.4 |
| Branched chain alpha-keto acid dehydrogenase | MWL092a07_r | 2.6 | 5.0 |
| Seed inhibition protein, putative | MWL069f08_r | 2.5 | 3.4 |
| Annexin | MDP065h05_f | 2.4 | 4.4 |
| Asparagine synthetase | GN021f11 | 2.4 | 30.2 |
| Nodule-enhanced sucrose synthase | MWL080c04_r | 2.3 | 4.8 |
| Annexin | MDP042e01_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) | GENL025e04 | 0.16 | 0.11 |
| Histidine decarboxylase | GEN054a02 | 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 | GN046g09 | 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 | MPDL062e05_f | 0.22 | 0.13 |
| HSP100/CIPB | MRL022d06_f | 0.23 | 0.15 |
| Syringolide-induced protein B13-1-9 | MWM033e05_r | 0.23 | 0.14 |
| Histidine decarboxylase, putative | MWL231b10_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 |

**Table 5. Continued**

| Current annotation | Gene ID | Gm6/SC3 | Nod |
|--------------------|---------|---------|-----|
| Ribonuclease non-S | MWL082g02_r | 0.26 | 0.31 |
| Putative zinc finger POZ protein | MWM026d08_r | 0.26 | 0.09 |
| WRKY transcription factor | GENL072f04 | 0.16 | 0.30 |
| Phenylalanine ammonia-lyase (LjPAL2) | GENL058e04 | 0.17 | 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 | GN020b04 | 0.29 | 0.20 |
| Cytochrome P450, putative | MR076i02_f | 0.30 | 0.06 |
| Putative anthocyanidine rhamnosyl-transferase | GN060a01 | 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.

130 Transcriptional Changes in *L. japonicus* AM Roots [Vol. 14,
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. 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, 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, 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. 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 aseptical 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.

Acknowledgements: We wish to thank Drs. K. Akiyama, Osaka Prefecture University, and Y. Tamada, Kyoto University, for the valuable discussions. We also thank Drs. C. L. Diaz-Argueta (Leiden University), K. Nagashima (Idemitsu Kosan Co.), and K. Nakamori (Kyoto University) for providing the protocol for hairy root transformation with *A. rhizogenes* LBA1334, the kind gift of the *G. mosseae* inoculum, and the instructions for embedding AM roots in plastic resin, respectively. This work was supported in part by an AIST Research Grant and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government. The array experiments were carried out at the Radioisotope Research Center, Kyoto University.

Supplementary Data: Supplementary data are available at www.dnaresearch.oxfordjournals.org

References

1. Schussler, A., Schwarzott, D. and Walker, C. 2001, A new fungal phylum, the *Glomeromycota*: phylogeny and evolution, *Mycol. Res.*, **105**, 1413–1421.
2. Smith, S. E. and Read, D. J. 1997, *Mycorrhizal Symbiosis*, 2nd Ed., Academic Press: San Diego.
3. Harrison, M. J. 2005, Signaling in the arbuscular mycorrhizal symbiosis, *Annu. Rev. Microbiol.*, **59**, 19–42.
4. Brundrett, M. 1991, Mycorrhizas in natural ecosystem, *Adv. Ecol. Res.*, **21**, 171–313.
5. Marschner, H. 1995, *Mineral Nutrition of Higher Plants*, 2nd Ed., Academic Press: San Diego, pp. 566–595.
6. Glaninazzi-Pearson, V. 1996, Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis, *Plant Cell*, **8**, 1871–1883.
7. Pirozynski, K. A. and Malloch, D. W. 1975, The origin of land plants: a matter of mycotrophism, *Biosystems*, **6**, 153–164.
8. Remy, W., Taylor, T. N., Hass, H. and Kerp, H. 1994, Four hundred-million-year-old vesicular arbuscular mycorrhizae, *Proc. Natl. Acad. Sci. USA*, **91**, 11841–11843.
9. Redeker, D., Kodner, R. and Graham, L. 2000, Glomalean fungi from the Ordovician, *Science*, **289**, 1920–1921.
10. Kistner, C., Winzer, T., Pitzschke, A., et al. 2005, Seven *Lotus japonicus* genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis, *Plant Cell*, **17**, 2217–2229.
11. Kistner, C. and Parniske, M. 2002, Evolution of signal transduction in intracellular symbiosis, *Trends Plant Sci.*, **7**, 511–518.
12. Journet, E. P., van Tuinen, D., Gouzy, J., et al. 2002, Exploring root symbiotic programs in the model legume Medicago truncatula using EST analysis, Nucleic Acids Res., 30, 5579–5592.

13. Wulf, A., Manthey, K., Doll, J., et al. 2003, Transcriptional changes in response to arbuscular mycorrhiza development in the model plant Medicago truncatula, Mol. Plant Microbe Interact., 16, 306–314.

14. Brechenmacher, L., Weidmann, S., van Tuinen, D., et al. 2004, Expression profiling of up-regulated plant and fungal genes in early and late stages of Medicago truncatula-Glomus mosseae interactions, Mycorrhiza, 14, 253–262.

15. Frenzel, A., Manthey, K., Perlick, A. M., et al. 2005, Combined transcriptome profiling reveals a novel family of arbuscular mycorrhizal-specific Medicago truncatula lectin genes, Mol. Plant Microbe Interact., 18, 771–782.

16. Liu, J., Blaylock, L. A., Endre, G., et al. 2003, Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis, Plant Cell, 15, 2106–2123.

17. Manthey, K., Krajinski, F., Hohnjec, N., et al. 2004, Transcriptome profiling in root nodules and arbuscular mycorrhiza identifies a collection of novel genes induced during Medicago truncatula root endosymbioses, Mol. Plant Microbe Interact., 17, 1063–1077.

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

19. Massoumou, M., van Tuinen, D., Chatagnier, O., et al. 2007, Medicago truncatula gene responses specific to arbuscular mycorrhiza interactions with different species and genera of Glomeromycota, Mycorrhiza, 17, 223–234.

20. Liu, J., Malkonado-Mendoza, I., Lopez-Meyer, M., Cheung, F., Town, C. D. and Harrison, M. J. 2007, Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots, Plant J., 50, 529–544.

21. Barker, D. G., Bianchi, S., Blondon, F., et al. 1990, Medicago truncatula, a model plant for studying the molecular genetics of the Rhizobium-legume symbiosis, Plant Mol. Biol. Rep., 8, 40–49.

22. Cook, D. R. 1999, Medicago truncatula—a model in the making! Curr. Opin. Plant Biol., 2, 301–304.

23. Salzer, P., Bonanomi, A., Beyer, K., et al. 2000, Differential expression of eight chitinase genes in Medicago truncatula roots during mycorrhiza formation, nodulation, and pathogen infection, Mol. Plant Microbe Interact., 13, 763–777.

24. Handberg, K. and Stougaard, J. 1992, Lotus japonicus, an autogamous, diploid legume species for classical and molecular genetics, Plant J., 2, 487–496.

25. Akiyama, K., Matsuzaki, K. and Hayashi, H. 2005, Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi, Nature, 435, 824–827.

26. Kouchi, H., Shimomura, K., Hata, S., et al. 2004, Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, Lotus japonicus, DNA Res., 11, 263–274.
activity develop in leaves of Arabidopsis and soybean, *Plant J.*, 41, 831–844.

44. Liu, H., Trieu, A. T., Blaylock, L. A. and Harrison, M. J. 1998, Cloning and characterization of two phosphate transporters from *Medicago truncatula* roots: regulation in response to phosphate and to colonization by arbuscular mycorrhizal (AM) fungi, *Mol. Plant Microbe Interact.*, 11, 14–22.

45. Rausch, C., Daram, P., Brunner, S., et al. 2001, A phosphate transporter expressed in arbuscule-containing cells in potato, *Nature*, 414, 462–470.

46. Paszkowski, U., Kroken, S., Roux, C. and Briggs, S. P. 2002, Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis, *Proc. Natl. Acad. Sci. USA*, 99, 13324–13329.

47. Blee, K. A. and Anderson, A. J. 1996, Defense-related transcript accumulation in *Phaseolus vulgaris* L. colonized by the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith, *Plant Physiol.*, 110, 675–688.

48. Gao, L. L., Knogge, W., Delp, G., Smith, F. A. and Smith, S. E. 2004, Expression patterns of defense-related genes in different types of arbuscular mycorrhizal development in wild-type and mycorrhiza-defective mutant tomato, *Mol. Plant Microbe Interact.*, 17, 1103–1113.

49. Harrison, M. J. and Dixon, R. A. 1993, Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular-arbuscular mycorrhizal associations in roots of *Medicago truncatula*, *Mol. Plant Microbe Interact.*, 6, 643–654.

50. Harrison, M. J. and Dixon, R. A. 1994, Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interactions between roots of *Medicago truncatula* and the mycorrhizal fungus *Glomus versiforme*, *Plant J.*, 6, 9–20.

51. Volpin, H., Elkind, Y., Okon, Y. and Kapulnik, Y. 1994, A vesicular arbuscular mycorrhizal fungus (*Glomus intraradices*) induces a defense response in alfalfa roots, *Plant Physiol.*, 104, 683–689.

52. Volpin, H., Phillips, D. A., Okon, Y. and Kapulnik, Y. 1995, Suppression of an isoflavonoid phytoalexin defense response in micorrhizal alfalfa roots, *Plant Physiol.*, 108, 1449–1454.

53. Lange, J., Xie, Z. P., Broughton, W. J., Vogeli-Lange, R. and Boller, T. 1999, A gene encoding a receptor-like protein kinase in the roots of common bean is differentially regulated in response to pathogens, symbionts and nodulation factors, *Plant Sci.*, 142, 133–145.

54. Montesano, M., Koiv, V., Mae, A. and Palva, E. T. 2001, Novel receptor-like protein kinases induced by *Erwinia carotovora* and short oligogalacturonides in potato, *Mol. Plant Pathol.*, 2, 339–346.

55. Logemann, E., Parniske, M. and Hahlbrock, K. 1995, Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley, *Proc. Natl. Acad. Sci. USA*, 92, 5905–5909.

56. Ulker, B. and Somssich, I. E. 2004, WRKY transcription factors: from DNA binding towards biological function, *Curr. Opin. Plant Biol.*, 7, 491–498.

57. Yamaguchi-Shinozaki, K. and Shinozaki, K. 1993, The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of rd22, a gene responsive to dehydration stress in *Arabidopsis thaliana*, *Mol. Gen. Genet.*, 238, 17–25.

58. Estabrook, E. M. and Sengupta-Gopalan, C. 1991, Differential expression of phenylalanine ammonia-lyase and chalcone synthase during soybean nodule development, *Plant Cell*, 3, 299–308.