DARWIN REVIEW

Molecular responses of *Lotus japonicus* to parasitism by the compatible species *Orobanche aegyptiaca* and the incompatible species *Striga hermonthica*

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Abstract

*Lotus japonicus* genes responsive to parasitism by the compatible species *Orobanche aegyptiaca* and the incompatible species *Striga hermonthica* were isolated by using the suppression subtractive hybridization (SSH) strategy. *O. aegyptiaca* and *S. hermonthica* parasitism specifically induced the expression of genes involved in jasmonic acid (JA) biosynthesis and phytoalexin biosynthesis, respectively. Nodulation-related genes were almost exclusively found among the *Orobanche*-induced genes. Temporal gene expression analyses revealed that 19 out of the 48 *Orobanche*-induced genes and 5 out of the 48 *Striga*-induced genes were up-regulated at 1 dai. Four genes, including putative trypsin protease inhibitor genes, exhibited systemic up-regulation in the host plant parasitized by *O. aegyptiaca*. On the other hand, *S. hermonthica* attachment did not induce systemic gene expression.

Key words: Host response, *Lotus japonicus*, *Orobanche*, *Striga*, suppression subtractive hybridization.

Introduction

*Orobanche* and *Striga* spp. are obligate root parasitic plants that affect the production of several agronomically important crops in many parts of the world. Among *Orobanche* spp., *O. aegyptiaca* and *O. ramosa* have the widest host range, including plants belonging to the following families: Solanaceae, Fabaceae, Brassicaceae, Cucurbitaceae, Asteraceae, Umbelliferae, Cannabinaceae, and Linaceae (Goldwasser and Kleifeld, 2004). *Striga* spp. exhibit great diversity in the semi-arid grasslands of Africa where three wide-ranging species, namely, *S. asiatica*, *S. gesnerioides*, and *S. hermonthica* are serious agronomic pests (Musselman et al., 1983). Of these three species, *S. hermonthica* mainly parasitizes tropical cereal crops and is the most devastating root parasite in Africa (Berner et al., 1995). The ultimate method for control of parasitic plants lies in the development of crops that are resistant to or tolerant toward such parasites. Although an entirely resistant or tolerant variety has not been identified or created thus far (Mohamed et al., 2003; Rubiales, 2003), information on host and non-host responses to parasitic plants has been accumulating at the molecular level. Studies based on the β-glucuronidase (GUS) strategy have revealed that *O. aegyptiaca* parasitism locally activates genes encoding the following proteins; a basic pathogenesis-related (PR) protein (Joel and Portnoy, 1998), 3-hydroxy-3-methylglutaryl CoA reductase 2 (Westwood et al., 1998), phenylalanine ammonia lyase, chalcone synthase, sesquiterpene cyclase, and farnesyltransferase in *Nicotiana tabacum* and 3-hydroxy-3-methylglutaryl CoA reductase 1 in *Lycopersicon esculentum* (Griffitts et al., 2004). Gowda et al. (1999) used a differential display strategy and isolated 23 genes whose expressions are up-regulated in the roots of *Tagetes erecta* during invasion by the incompatible *S. asiatica*. One of these up-regulated genes, i.e., the non-host resistance to *S. asiatica* (NRSA-1)
gene, encodes a protein that is highly homologous to the disease-resistance proteins identified in several plants. Using the suppression subtractive hybridization (SSH) strategy, genes were isolated from *Arabidopsis thaliana* roots inoculated with *O. ramosa* (Vieira-Dos-Santos et al., 2003b), *Medicago truncatula* roots inoculated with *O. crenata* (Die et al., 2007), and sorghum roots parasitized by *S. hermonthica* (Hiraoka and Sugimoto, 2008). For each experiment, genes involved in plant defence response mechanisms such as the jasmonic acid (JA) pathway, signal transduction, and cell-wall fortification were isolated.

Recently, Kubo et al. (2008) reported that *L. japonicus* is a suitable host for the study of parasitism in plants. This model legume is compatible to *O. aegyptiaca* and incompatible to *O. minor*, *S. gesnerioides*, and *S. hermonthica*, of which only *S. hermonthica* induces tissue-browning of *L. japonicus* at the attachment sites. Nearly 700,000 nucleotide sequences representing the Fabaceae are available from the National Center for Biotechnology Information (NCBI) (Graham et al., 2004), and functional genomic studies have been carried out on the model legumes including *L. japonicus* (VandenBosch and Stacey, 2003). The Institute for Genomic Research (TIGR) has analysed expressed sequence tags (ESTs) from a variety of plant species, including *L. japonicus*, and clustered the ESTs into tentative consensus sequences (TCs) that represent the minimally redundant set of a species’ expressed genes (http://www.tigr.org/tdb/tgi/plant.shtml). In this study, two subtracted cDNA libraries were constructed, namely, Lj-Oa and Lj-Sh, by using SSH (Diatchenko et al., 1996). Lj-Oa and Lj-Sh were enriched for *L. japonicus* genes that were up-regulated in response to parasitism by *O. aegyptiaca* and *S. hermonthica*, respectively. Changes in the temporal and systemic expression of the genes were analysed in plants inoculated with *O. aegyptiaca* and *S. hermonthica* with the objective of gaining more comprehensive knowledge on both host and non-host responses to parasitic plants at the molecular level.

**Materials and methods**

**Plant materials and growth conditions**

Seeds of *L. japonicus* accession Miyakojima MG-20 were supplied by the National BioResource Project, Miyazaki University, Japan. *O. aegyptiaca* seeds collected from mature plants parasitizing *Vicia sativa* were provided by Professor J Scholes, The University of Sheffield, U.K. *S. hermonthica* seeds were obtained from Professor AGT Babiker, Sudan University of Science and Technology, Sudan. *L. japonicus* plants were grown in rhizotrons as described by Kubo et al. (2008).

**Split-root system**

For analyses of the systemic gene expression triggered in response to *O. aegyptiaca* and *S. hermonthica* parasitism, the split-root system as described by Koss-lak and Bohlool (1984) was employed with some modifications. A modified split-root system was developed using two square Petri dishes (height, 14.4 cm; width, 10.4 cm; thickness, 1.6 cm), filled with rockwool, and overlaid with glass fibre paper. This system was carefully designed to prevent any exchange of material between the dishes (Fig. 1).

*L. japonicus* plants grown for 2 weeks in test tubes were transplanted to the modified split-root system, and the roots of each plant were split into halves. The roots placed in one Petri dish were inoculated with *O. aegyptiaca* and *S. hermonthica* radicles and those in the other were uninoculated (Fig. 1).

**Conditioning and germination of *O. aegyptiaca* and *S. hermonthica* seeds and inoculation**

The seeds of *O. aegyptiaca* and *S. hermonthica* were surface-sterilized and conditioned as described by Kubo et al. and inoculated.
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(2008) and Sugimoto et al. (2003), respectively. Seed germination was induced using GR24, a synthetic stimulant provided by Professor B Zwanenburg, Nijmegen University, The Netherlands. Radicles of *O. aegyptiaca* and *S. hermonthica* were inoculated onto the *L. japonicus* roots in the manner described by Kubo et al. (2008).

At 10 d and 6 d after inoculation (dai), 10 mm long root segments were excised 5 mm from the inoculation sites of *O. aegyptiaca* and *S. hermonthica*, respectively, and were used for SSH. For analyses of the systemic gene expression, 40 radicles each of *O. aegyptiaca* and *S. hermonthica* were placed onto the roots in one dish of the modified split-root system at 2 weeks after transplantation. The roots and the leaves in the other uninoculated dish were excised at 1, 2, and 10 dai of *O. aegyptiaca* and 1, 2, and 6 dai of *S. hermonthica*. The roots and leaves from uninoculated plants were collected as control samples. The excised roots and leaves were immediately frozen in liquid nitrogen and stored at –80 °C until use.

**Suppression subtractive hybridization (SSH)**

Total RNA of *L. japonicus* was isolated from the *O. aegyptiaca*-parasitized roots at 10 dai, *S. hermonthica*-attached roots at 6 dai, and the uninoculated roots using the RNase plant mini kit (Qiagen); synthesis of the first and second cDNA strands was performed from 60, 300, and 300 ng total RNA, respectively, using the Clontech SMART PCR cDNA synthesis kit (Clontech). SSH was performed using the Clontech PCR-Select cDNA subtraction kit (Clontech). To construct the Lj-Oa library containing *L. japonicus* genes up-regulated in response to parasitism by *O. aegyptiaca*, cDNAs obtained from the *O. aegyptiaca*-parasitized roots and the uninoculated roots were used as the tester and the driver cDNAs for SSH, respectively. Similarly, to construct the Lj-Sh library containing genes up-regulated in response to parasitism by *S. hermonthica*, cDNAs obtained from the *S. hermonthica*-attached roots and the uninoculated roots were used as the tester and the driver cDNAs, respectively. The secondary PCR products were cloned and sequenced and the redundant clones were eliminated as described previously (Hiraoka and Sugimoto, 2008). A database search was performed for each sequence by using the BLASTN, BLASTX, and TBLASTX programs in NCBI and TIGR databases, with E values of ≤1.

**Expression analysis of the subtracted cDNAs**

The expression of the subtracted cDNAs was analysed by performing quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using gene-specific primers designed on the basis of each cDNA sequence. Total RNA was extracted from each sample using the RNase plant mini kit. The DNase treatment of each total RNA, cDNA synthesis, and qRT-PCR analysis were performed as described previously (Hiraoka and Sugimoto, 2008). For one qRT-PCR cycle, a cDNA sample equivalent to 0.5 ng of total RNA was used as the template. The values obtained were normalized to those obtained in the case of actin (accession number EU195536), which was used as an internal control and has been confirmed to exhibit similar expression levels under the test conditions. Each experiment was conducted in triplicate. Of the genes isolated by SSH, those exhibiting greater than 2-fold up-regulation were selected by performing qRT-PCR and were deposited in the DNA Data Bank of Japan (DDBJ) database under the accession numbers BB998881 to BB999976.

**Results**

**Isolation of genes up-regulated in response to parasitism by *O. aegyptiaca* and *S. hermonthica* and temporal changes in the expression of these genes**

*O. aegyptiaca* tubercle formation and tissue browning at the attachment sites of *S. hermonthica*, as reported by Kubo et al. (2008), were observed on *L. japonicus* roots at 10 dai and 6 dai, respectively. These roots were employed in SSH for constructing the subtracted cDNA libraries Lj-Oa and Lj-Sh. Lj-Oa and Lj-Sh comprised 297 and 336 colonies, respectively, containing the PCR product inserts. After eliminating redundancy, 116 Lj-Oa colonies and 89 Lj-Sh colonies were selected. The expression levels of all the Lj-Oa genes in *O. aegyptiaca*-parasitized roots and uninoculated roots were compared by performing qRT-PCR, and 48 genes that exhibited greater than 2-fold up-regulation were identified as *Orobanche*-induced genes. Similarly, 48 Lj-Sh genes were identified as *Striga*-induced genes. No over-lapping nucleotide sequence was detected in the *Orobanche-* and the *Striga*-induced genes.

Temporal changes in the expression levels of the *Orobanche*-induced genes in the *L. japonicus* roots were evaluated at 1, 2, and 10 dai. Similarly, the expression levels of the *Striga*-induced genes in the roots were evaluated at 1, 2, and 6 dai. On the basis of the expression at 1 dai, all the genes were classified into three clusters as shown in Figs 2 and 3. Clusters I, II, and III comprised genes that exhibited up-regulation, constant expression, and down-regulation, respectively, at 1 dai. Of the *Orobanche*-induced genes, 19, 26, and 3 genes were classified into clusters I, II, and III, respectively (Fig. 2). On the other hand, 6, 33, and 9 of the *Striga*-induced genes were classified into these respective clusters (Fig. 3). Of the genes in cluster I, the expression levels of 11 *Orobanche*-induced genes and three *Striga*-induced genes were similar to those in uninoculated roots at 2 dai (Figs 2, 3), and those in LjOa116-3s and LjOa25 exhibited transient down-regulation at 2 dai (Fig. 2). In the clusters II and III, the expression of most *Orobanche*- and *Striga*-induced genes was up-regulated only at 10 and 6 dai, respectively (Figs 2, 3).

**Functional categories of up-regulated genes**

On the basis of their functions suggested by the homology search, all the *Orobanche-* and *Striga*-induced genes were classified into 11 categories (Tables 1, 2). JA biosynthesis-
and phytoalexin biosynthesis-related genes were exclusively included in Lj-Oa and Lj-Sh, respectively (Tables 1, 2). The JA biosynthesis-related genes LjOa25, LjOa83-1, and LjOa74 were found to be homologous to the lipoxygenase (LOX)-encoding genes of *Pisum sativum*, *Cicer arietinum*, and *Sesbania rostrata*, respectively (Table 1). Further, the phytoalexin biosynthesis-related genes LjSh207, LjSh46-1, and LjSh29s were identical to those encoding isoflavone
| Clone        | Homology (species; accession number)                                                                 | E-value | Accession no. |
|--------------|-------------------------------------------------------------------------------------------------------|---------|---------------|
| Jasmonic acid biosynthesis                           |                                                      |         |               |
| LjOa25       | Lipoxigenase (Pisum sativum; Q24470)                                                                  | 3.8E-66 | BB999902      |
| LjOa83-1     | Lipoxigenase (Cicer arietinum; Q9M325)                                                                | 2.0E-84 | BB999909      |
| LjOa74       | Lipoxigenase mRNA (Sesbania rostrata; AJ309069)                                                       | 2.0E-73 | BB999926      |
| Nodulation related                                   |                                                      |         |               |
| LjOa196      | Anti-H(I) lectin (Lotus tetragonolobus; P196644)                                                     | 4.1E-112| BB999884      |
| LjOa85s      | Anti-H(I) lectin (Lotus tetragonolobus; P196644)                                                     | 7.1E-49 | BB999891      |
| LjOa51-2     | Nod factor binding lectin-nucleotide phosphohydrolase mRNA (Lotus japonicus; AF156780)                | 2.0E-93 | BB999889      |
| LjOa95       | Repetitive proline-rich cell wall protein 2 precursor (Medicago truncatula; Q40375)                   | 2.8E-46 | BB999897      |
| LjOa109      | EST generated from nodules of 5- and 7-week-old plants (Lotus japonicus; CB827466)                    | 5.10E-107| BB999894      |
| LjOa60-1     | MIN19-like protein (Pisum sativum; AAU14999)                                                         | 1.0E-11 | BB999918      |
| LjOa157-1    | Actin-depolymerizing factor 2 (Petunia×hybrida; Q9FV11)                                              | 3.8E-84 | BB999921      |
| Pathogenesis related                                 |                                                      |         |               |
| LjOa9        | Miraculin-like protein (Solanum breviflorum; CAOO6377)                                               | 1.1E-121| BB999888      |
| LjOa169      | Serine proteinase inhibitor (Medicago sativa; Q40329)                                                | 1.6E-27 | BB999890      |
| LjOa148      | Ripening-related protein (Pisum sativum; AAQ72568)                                                   | 1.2E-16 | BB999883      |
| LjOa162-1    | Ripening-related protein (Pisum sativum; AAQ72568)                                                   | 1.7E-43 | BB999885      |
| LjOa6        | Serine proteinase inhibitor (Medicago sativa; Q40329)                                                | 1.4E-24 | BB999892      |
| LjOa124-1    | Protease inhibitor/seed storage/lipid transfer protein family protein (Arabidopsis thaliana; NP_565872)| 5.0E-31 | BB999896      |
| LjOa26-2     | Thaumatin-like protein PR-5b precursor (Cicer arietinum; Q81926)                                      | 1.1E-40 | BB999893      |
| LjOa58-2     | Serine proteinase inhibitor (Medicago sativa; Q40329)                                                | 5.6E-20 | BB999898      |
| LjOa135s     | Ripening-related protein (Pisum sativum; AAQ72568)                                                   | 1.7E-121| BB999900      |
| LjOa181      | Cysteine proteinase inhibitor mRNA (Glycine max; U51855)                                              | 9.0E-05 | BB999910      |
| LjOa28       | Protease inhibitor (Glycine max; Q96807)                                                              | 1.6E-111| BB999899      |
| LjOa214      | Bowman-birk type proteinase inhibitor (Amburana acreana; P83284)                                     | 2.1E-97 | BB999901      |
| LjOa286      | Serine proteinase inhibitor (Medicago sativa; Q40329)                                                | 3.8E-36 | BB999912      |
| LjOa5227     | Pathogenesis-related protein 2 (Phaseolus vulgaris; P25986)                                           | 7.0E-130| BB999913      |
| LjOa217-1    | PR10-1 protein (Medicago truncatula; P93333)                                                         | 1.2E-73 | BB999915      |
| Growth       |                                                      |         |               |
| LjOa147-2    | Flavonol 3-sulphotransferase (Flaveria bidentis; P52835)                                             | 1.5E-22 | BB999887      |
| LjOa145      | S-Adenosylmethionine decarboxylase proenzyme (Vicia faba; Q6M4D8)                                    | 1.7E-61 | BB999903      |
| LjOa147-1    | Asparagine synthase (Lotus japonicus; CAA61590)                                                      | 4.5E-76 | BB999905      |
| LjOa215      | Putative phytosulphokine peptide precursor mRNA (Glycine max; BK000118)                              | 3.0E-20 | BB999886      |
| LjOa226-1    | Histidine amino acid transporter (Oryza sativa; CAD98902)                                            | 6.0E-24 | BB999916      |
| LjOa33       | Putative cytidine or deoxycytidylic deaminase mRNA (Cicer arietinum; AJ006764)                        | 4.8E-144| BB999917      |
| LjOa60-2     | Steroid sulfotransferase-like protein (Arabidopsis thaliana; Q8L5A7)                                 | 0.52    | BB999908      |
| Defence response                                     |                                                      |         |               |
| LjOa159      | ERD15 protein (dehydration-induced protein) (Arabidopsis thaliana; Q39096)                           | 1.8E-91 | BB999919      |
| LjOa58-1     | Probable flavin-containing monoxygenase 1 (Arabidopsis thaliana; Q9MLA1)                            | 4.0E-14 | BB999907      |
| LjOa40-1     | Resistant specific protein-1(4) (Vigna radiata; QBGSG3)                                             | 0.087   | BB999927      |
| LjOa62       | Lipid transfer protein precursor (Pisum sativum; AAF61436)                                          | 2.0E-40 | BB999924      |
| Cell-wall fortification                              |                                                      |         |               |
| LjOa165-2    | Glycine-rich protein (Arabidopsis thaliana; NP_565380)                                              | 3.0E-06 | BB999881      |
| LjOa143      | Putative cinnamyl alcohol dehydrogenase (Oryza sativa; Q8H859)                                     | 1.5E-41 | BB999904      |
| LjOa40-2     | Peroxidase precursor (Vigna angularis; Q43854)                                                      | 1.0E-67 | BB999928      |
| Detoxification of reactive oxygen species             |                                                      |         |               |
| LjOa141-1    | NADH dehydrogenase ND6 (Lotus japonicus; BAB33248)                                                  | 0.77    | BB999922      |
| Other function                                       |                                                      |         |               |
| LjOa110-2    | Putative phosphatase (Glycine max; Q8GT55)                                                           | 3.7E-71 | BB999906      |
| Unknown functions                                     |                                                      |         |               |
| LjOa116-3s   | Prion-like-(q/n-rich)-domain-bearing protein 75, isoform a (Caenorhabditis elegans; AAC48255)        | 0.34    | BB999882      |
| LjOa88       | PGPS/D10 (Petunia×hybrida; Q9ZTM9)                                                                   | 1.4E-111| BB999911      |
| LjOa24       | UV/1 (Pisum sativum; Q9AULH7)                                                                       | 4.5E-08 | BB999920      |
| LjOa163      | UV/1 (Pisum sativum; Q9AULH7)                                                                       | 1.2E-07 | BB999914      |
| LjOa51-1     | Unknown protein (Populus trichocarpa; ABK94704)                                                     | 0.001   | BB999923      |
| LjOa146      | Unnamed protein (Vitis vinifera; CAD44822)                                                          | 0.004   | BB999925      |
| No homology                                          |                                                      |         |               |
| LjOa162-2    | None                                                  | –       | BB999895      |
Table 2. Genes showing up-regulated expression in the roots of *Lotus japonicus* after 6 d of *Striga hermonthica* inoculation

| Clone          | Homology (species; accession number)                                                                 | E-value     | Accession no. |
|----------------|-------------------------------------------------------------------------------------------------------|-------------|---------------|
| **Phytoalexin biosynthesis**        |                                                                                                         |             |               |
| LjSh207-1      | Isoflavone reductase homologue mRNA R7 (*Lotus japonicus*; AB265595)                                     | 0           | BB999944      |
| LjSh46-1       | Pinosylvin-lariciresinol reductase homologue R5 mRNA (*Lotus japonicus*; AB265593)                      | 1.8E-14     | BB999951      |
| LjSh29s        | Cytochrome P450 mRNA (*Lotus japonicus*; AB025016)                                                    | 1.4E-13     | BB999933      |
| **Nodulation related**               |                                                                                                         |             |               |
| LjSh1s         | Small GTP-binding protein RAB11I mRNA (*Lotus japonicus*; Z73957)                                      | 3.0E-81     | BB999929      |
| **Pathogenesis related**              |                                                                                                         |             |               |
| LjSh70-2       | Miraculin-like protein (*Soluranum brevidens*; AAQ96377)                                              | 8.8E-17     | BB999940      |
| LjSh76-2       | Class I chitinase (*Medicago sativa*; P49084)                                                        | 3.8E-75     | BB999930      |
| LjSh232-1      | Pathogenesis-related protein 2 (*Phaseolus vulgaris*; P25986)                                         | 1.1E-39     | BB999945      |
| LjSh239-2      | PR10-1 protein (*Medicago truncatula*; P93333)                                                       | 2.0E-75     | BB999931      |
| LjSh201-1      | Pathogenesis-related protein 2 (*Phaseolus vulgaris*; P25986)                                         | 1.2E-39     | BB999961      |
| **Growth**     |                                                                                                         |             |               |
| LjSh269-2      | Asparagine synthase-related protein (*Elaeis guineensis*; AAT76902)                                   | 1.0E-52     | BB999936      |
| LjSh207-2      | Putative SKP1-like protein (*Orzya sativa*; Q8GWV5)                                                 | 2.0E-25     | BB999938      |
| LjSh251-2      | Putative SKP1-like protein (*Orzya sativa*; Q8GWV5)                                                 | 6.4E-88     | BB999939      |
| LjSh251-1      | Hexose carrier (*Ricinus communis*; Q41139)                                                          | 1.9E-65     | BB999941      |
| LjSh153        | Asparagine synthase-related protein (*Elaeis guineensis*; AAT76902)                                   | 9.0E-66     | BB999950      |
| LjSh263-2      | Thioesterase FatA1 (*Cuphea hookeriana*; Q9T7F7)                                                     | 0.049       | BB999946      |
| LjSh109-1      | 60S ribosomal protein L7a-1 (*Arabidopsis thaliana*; P49692)                                         | 2.8E-19     | BB999952      |
| LjSh56-1       | Alpha galactosidase precursor (*Coffea arabica*; CAJ40777)                                          | 3.0E-09     | BB999953      |
| LjSh269s       | Acetyl-CoA acetyltransferase (*Cucumis sativus*; Q03875)                                            | 3.1E-81     | BB999954      |
| LjSh104-1      | Phosphoserine aminotransferase (*Arabidopsis thaliana*; Q8L7P0)                                     | 5.0E-14     | BB999969      |
| LjSh82         | 40S ribosomal protein S30 (*Arabidopsis thaliana*; P49698)                                           | 1.0E-09     | BB999971      |
| LjSh10s        | Suspensor-specific protein (*Phaseolus coccineus*; AA14318)                                         | 1.0E-22     | BB999956      |
| LjSh183-1      | Lysine histidine transporter 1 (*Arabidopsis thaliana*; NP_851109)                                   | 9.0E-36     | BB999967      |
| LjSh239-1      | Ubiquitin-conjugation enzyme (*Glycine max*; Q8LJR9)                                                 | 2.8E-58     | BB999976      |
| **Defence response**                  |                                                                                                         |             |               |
| LjSh49-1       | Putative 1-aminoacyclopropane-1-carboxylate oxidase (*Arabidopsis thaliana*; Q43383)                   | 3.1E-32     | BB999932      |
| LjSh70-1       | 12-oxophytodienoic acid 10, 11-reductase (*Pisum sativum*; BAD12184)                                 | 3.0E-34     | BB999942      |
| LjSh72-1s      | Disease resistance protein-related / LRR protein-related (*Arabidopsis thaliana*; NP_564426)           | 4.0E-16     | BB999943      |
| LjSh171-1s     | S-Adenosylmethionine synthase mRNA (*Medicago sativa*; AY560003)                                      | 0           | BB999948      |
| LjSh68         | Dehydrin-like protein (*Soluranum sogaranium*; Q869E7)                                              | 5.2E-63     | BB999968      |
| LjSh83-1       | Heat shock cognate protein 71.0 (*Pisum sativum*; Q41027)                                            | 3.5E-46     | BB999970      |
| LjSh156s       | Phosphatidylinositol 4-kinase (*Arabidopsis thaliana*; CAB37928)                                      | 3.0E-06     | BB999975      |
| LjSh244s       | Dehydrin (*Phaseolus vulgaris*; Q41111)                                                               | 1.7E-46     | BB999969      |
| **Cell-wall fortification**           |                                                                                                         |             |               |
| LjSh104-2      | Cinnamyl alcohol dehydrogenase-like protein gene (*Lotus comiculatus*; AY028929)                      | 1.1E-14     | BB999949      |
| LjSh269-1      | Putative cinnamyl alcohol dehydrogenase (*Orzya sativa*; Q8H859)                                      | 1.3E-29     | BB999958      |
| **Detoxification of reactive oxygen species** |                                                                                                         |             |               |
| LjSh7         | Phospholipid hydroperoxide glutathione peroxidase (*Momordica charantia*; Q8W259)                     | 7.3E-42     | BB999947      |
| LjSh182-1      | Homogentisic acid geranylgeranyl transferase (*Triticum aestivum*; Q7XB13)                           | 3.0E-66     | BB999965      |
| LjSh162s       | Catalase 1b mRNA (*Lotus japonicus*; AY424952)                                                       | 0           | BB999974      |
| LjSh60         | Glutathione S-transferase 7 mRNA (*Glycine max*; AF243662)                                           | 2.0E-52     | BB999973      |
| **Other functions**                   |                                                                                                         |             |               |
| LjSh144        | Snip25a (*Arabidopsis thaliana*; AAM02553)                                                           | 2.7E-12     | BB999963      |
| LjSh66-1s      | Kruppel like factor 4-like mRNA (*Dianio rerio*; AM422104)                                           | 0.0002      | BB999964      |
| LjSh7-1        | Serine/threonine-protein kinase tel1 (*Schizosaccharomyces pombe*; O74630)                           | 0.18        | BB999962      |
| **Unknown functions**                 |                                                                                                         |             |               |
| LjSh86-1       | Coronin binding protein (*Dictyostelium discoideum*; O61085)                                        | 0.0011      | BB999937      |
| LjSh132        | Uncharacterized Cys-rich domain (*Medicago truncatula*; ABD322901)                                   | 0.007       | BB999957      |
| LjSh15         | Integral membrane family protein (*Arabidopsis thaliana*; NP_567472)                                 | 1.6E-46     | BB999934      |
| LjSh76-1       | Uncharacterized Cys-rich domain (*Medicago truncatula*; ABD322901)                                   | 7.0E-14     | BB999960      |
| LjSh107s       | UPF0497 membrane protein (*Arabidopsis thaliana*; Q0SQU2)                                            | 2.0E-17     | BB999935      |
| LjSh11         | Integral membrane family protein (*Arabidopsis thaliana*; NP_567472)                                 | 3.1E-56     | BB999955      |
| LjSh290s       | UVI1 (*Pisum sativum*; Q9AUH7)                                                                      | 1.5E-54     | BB999972      |
| **No homology**                          |                                                                                                         |             |               |
| LjSh24-1       | None                                                                                                 | –           | BB999966      |
reductase (IFR), pinoresinol-lariciresinol reductase (PLR), and cytochrome P450 in *L. japonicus*, respectively (Table 2).

Seven genes involved in nodulation were included among the *Orobanche*-induced genes (Table 1). Both *LjOa198* and *LjOa85s* were found to be homologous to a lectin-encoding gene of *Lotus tetragonolobus*. *LjOa51-2* was identified as a gene encoding Nod factor-binding lectin-nucleotide phosphohydrolase (LNP). *LjOa95*, *LjOa60-1*, *LjOa157-1*, and *LjOa109* were determined to be homologous to the repetitive proline-rich cell-wall protein (PRP) 2 precursor of *M. truncatula*, the MtN19-like protein of *P. sativum*, actin-depolymerizing factor 2 of *Petunia hybrida*, and an EST generated from the nodules of 5- and 7-week-old *L. japonicus* plants, respectively (Table 1). The *Striga*-induced genes included only one gene involved in nodulation.

In the case of PR genes, nine out of the 15 in the *Orobanche*-induced genes were homologous to protease-inhibitor genes (Table 1). On the other hand, only one protease-inhibitor gene was included among the *Striga*-induced genes (Table 2).

**Systemic expression of up-regulated genes**

Genes were selected that exhibited greater than 8-fold up-regulation at either time point after the inoculation of *O. aegyptiaca* or *S. hermonthica* (Figs 4A, 5A), and their systemic expression was analysed (Figs 4B, 5B, 5C). Among 16 *Orobanche*-induced genes, four genes, namely, *LjOa9*, *LjOa116-3s*, *LjOa169*, and *LjOa147-2* exhibited greater than 8-fold up-regulation at 10 dai (Fig. 5B). The expression of these four genes in the leaves was also analysed, and a 10-fold up-regulation of *LjOa9* expression was detected at 10 dai (Fig. 5C). Similarly, the systemic expression of 14 genes selected from among the *Striga*-induced genes was analysed in the uninoculated roots at 1, 2, and 6 dai (Fig. 4B). However, no gene exhibited significant up-regulation (Fig. 4B).

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**Fig. 4.** Local (A) and systemic (B) expression of the 12 *Orobanche*-induced genes and the 14 *Striga*-induced genes in *Lotus japonicus* roots. The x-axis indicates the clone number and the days after inoculation of each gene. The y-axis indicates fold expression induction (log scale). The error bars represent the SD of the inductions.
The fact that no overlapping nucleotide sequence was detected between the *Orobanche*-induced genes and *Striga*-induced genes indicates that *L. japonicus* roots are able to distinguish the compatible parasite from the incompatible one. The expression of most of the *Striga*-induced genes in the inoculated roots was as low as that in the uninoculated roots at 1 dai and 2 dai. This delayed response is consistent with the phenomena of tissue browning, which was not observed at 1 dai or 2 dai but was evident at 6 dai of *S. hermonthica* (Kubo et al., 2008). On the other hand, the expression of approximately 40% (19 genes) of the *Orobanche*-induced genes was up-regulated at 1 dai of *O. aegyptiaca*. Considering that 13 out of the 19 genes exhibited up-regulation at 1 dai and 10 dai and down-regulation at 2 dai, the expression of these 13 genes may, therefore, have been induced at different stages of parasitism, namely, attachment and tubercle formation. These results are in accord with those of a previous study by Vieira-Dos-Santos et al. (2003b), wherein four out of the 13 Arabidopsis genes that were up-regulated by *O. ramosa* parasitism exhibited a second induction phase at 7 dai.

Genes encoding putative LOX were exclusively included among the *Orobanche*-induced genes. LOX oxidizes linolenic acid, and the resultant hydroperoxide can be a precursor of JA (Liechti and Farmer, 2002). Previous reports have also described the induction of genes related to JA biosynthesis in host plants parasitized by *O. ramosa* (Vieira-Dos-Santos et al., 2003a, b), *O. crenata* (Die et al., 2007), and *S. hermonthica* (Hiraoka and Sugimoto, 2008). It is well-known that JA mediates wound responses in plants (Mason and Mullet, 1990). Up-regulation of LOX gene expression is indicative of host root wounding by the parasite and the stress signal is transmitted via JA although it dose not elicit a rapid response (Fig. 2). This hypothesis is supported by a light microscopic study conducted by Kubo et al. (2008), which revealed that the *O. aegyptiaca* endophyte oppresses the *L. japonicus* vascular parenchyma, xylem, and phloem.

Interestingly, attachment of the incompatible *S. hermonthica* to the host roots induced the specific expression of genes encoding IFR, PLR, and cytochrome P450, which catalyse the late steps in the biosynthesis of vestitol, a legume-specific phytoalexin 5-deoxyisoflavonoid (Shimada et al., 2007). Vestitol accumulates in *L. corniculatus* in response to inoculation with the fungus *Helminthosporium turcicum* (Bonde et al., 1973). Induction of vestitol biosynthesis-related genes suggests that *L. japonicus* recognizes the incompatible *S. hermonthica* as an unfavourable intruder similar to pathogenic fungi, and it then synthesizes vestitol as a non-host resistance response to *S. hermonthica*. In a study on the response of *M. truncatula* to *O. crenata*, Lozano-Baena et al. (2007) demonstrated that phenolic compounds accumulate in infected host roots; however, neither the chemical structures nor the biological functions of these compounds have been identified to date. The above-mentioned authors postulated that the host poisons the parasite by releasing toxic metabolites through the vascular connections.

The fact that the genes involved in nodulation were almost exclusively found among the *Orobanche*-induced genes suggests that *L. japonicus* recognizes the compatible *O. aegyptiaca* as a symbiont similar to rhizobium. Among the seven nodulation-related genes, the putative lectin genes (*LjOa198* and *LjOa85s*) and LNP (*LjOa51-2*) exhibited up-regulation at 1 dai. In *Dolichos biflorus*, Db-LNP, which is expressed on the surface of young and emerging root hairs,
binds to the Nod factors produced by rhizobial strains that nodulate this plant (Roberts et al., 1999). Db-LNP is redistributed to the tips of the root hairs in response to root treatment with a rhizobial symbiont or with the Nod factor but not with a non-symbiotic rhizobial strain or a root pathogen (Kalsi and Etzler, 2000). The expression of LjOu95, which is homologous to MtPRP2, was also induced at 1 dai. MtPRP2 is important for remodelling of the host extracellular matrix, which is involved in the early response of legume host roots to rhizobia (Wilson et al., 1994). The four genes that exhibited up-regulated expression at 1 dai may play significant roles during the early stages of the parasitic establishment of O. aegyptiaca.

It is noteworthy that PR genes accounted for 31% of the Orobanche-induced genes and that more than half of the PR genes were up-regulated at 1 dai. In another compatible relationship between sorghum and S. hermonthicua, wherein the tubercle formation rate was high (>58%), only two PR genes were included among the 30 genes that were up-regulated by parasitism (Hiraoka and Sugimoto, 2008). A low rate of tubercle formation (<10%) may be attributable to the up-regulation of PR gene expression in L. japonicus following O. aegyptiaca attachment.

The phenomena of systemic induction of genes in response to plant parasitism are disputable. Gowda et al. (1999) reported that S. asiatica infection induces the systemic expression of NRSA-1 in the roots and leaves of T. erecta. On the other hand, no systemic gene induction was detected in N. tabacum and L. esculentum parasitized by O. aegyptiaca (Joel and Portnoy, 1998; Westwood et al., 1998; Griffitts et al., 2004). However, in the present study, it was observed that O. aegyptiaca parasitism induced the systemic expression of LjOu9, which is homologous to a miraculin-like protein; this demonstrated that wound-induced signal transduction was systemically induced in L. japonicus by O. aegyptiaca parasitism.

In summary, the L. japonicus genes that are up-regulated in response to parasitism by the compatible species O. aegyptiaca and the incompatible species S. hermonthicua were isolated. Our comparison between the Orobanche- and the Striga-induced genes with regard to their expression patterns and putative functions suggested that L. japonicus is likely to recognize the incompatible species S. hermonthicua as an unfavourable intruder. Moreover, Nod genes were induced following the attachment of the compatible species O. aegyptiaca to the host roots. Successful parasitism induced the expression of JA and PR genes, some of which were systemically expressed.

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