Journal of Experimental Botany, Vol. 60, No. 2, pp. 641–650, 2009
doi:10.1093/jxb/ern316 Advance Access publication 16 December, 2008
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

DARWIN REVIEW

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

Yukihiro Hiraoka, Hiroaki Ueda and Yukihiro Sugimoto*

Graduate School of Agricultural Science, Kobe University, Rokkodai, Nada, 657-8501 Kobe, Japan

Received 3 September 2008; Revised 3 November 2008; Accepted 12 November 2008

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)

* To whom correspondence should be addressed: E-mail: yukihiro@kobe-u.ac.jp
Abbreviations: dai, day(s) after inoculation; EST, expressed sequence tag; GUS, β-glucuronidase; IFR, isoflavone reductase; JA, jasmonic acid; LAR, localized acquired resistance; LNP, Nod factor-binding lectin-nucleotide phosphohydrolase; LOX, lipoxygenase; MeJA, methyl jasmonate; PLR, pinoresinol-lariciresinol reductase; PR protein, pathogenesis-related protein; PRP, proline-rich cell-wall protein; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SA, salicylic acid; SSH, suppression subtractive hybridization.
© 2008 The Author(s).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

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.*, 2003*b*), *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, UK. *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 Kosslak 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.*

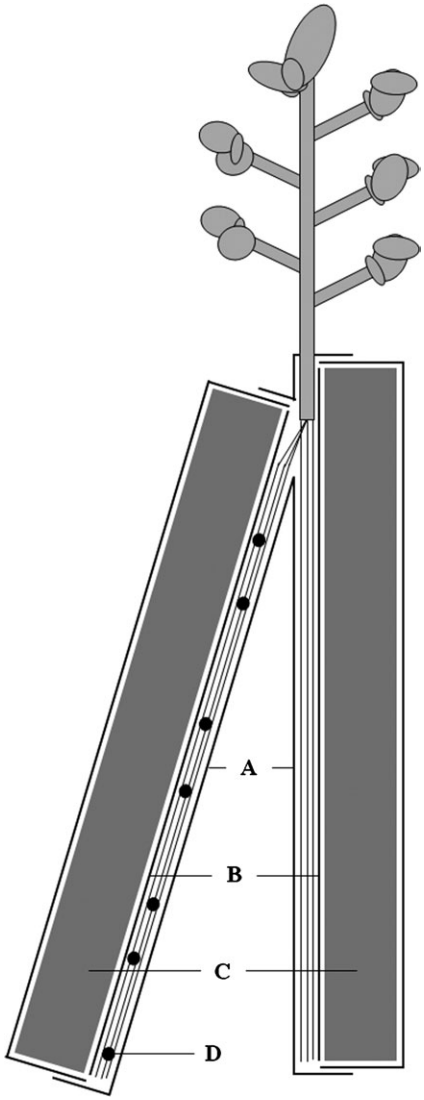

Fig. 1. Diagram of the modified split-root assembly. (A), square Petri dishes; (B), glass fibre papers; (C), rockwool; (D), *Orobanche aegyptiaca* or *Striga hermonthica* inoculated on one half of the *Lotus japonicus* roots.

(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 RNeasy 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 $\leqslant 1$.

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 RNeasy 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 BB999881 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 overlapping 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-

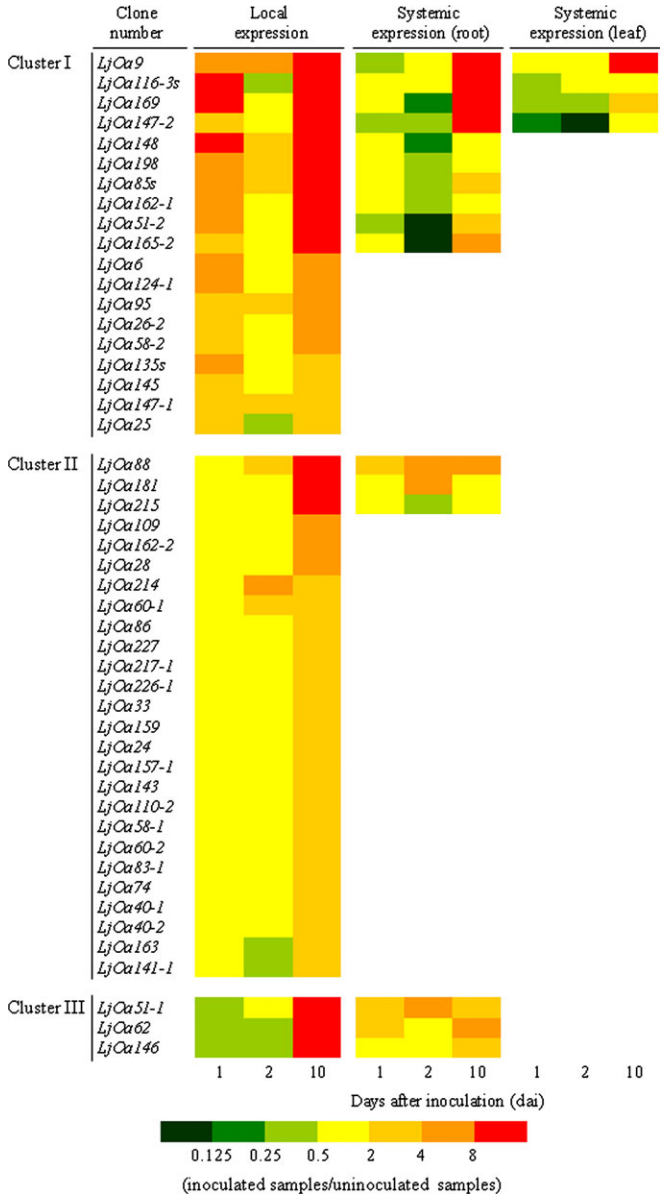

Fig. 2. Expression profiles of *Lotus japonicus* genes that were up-regulated in response to *Orobanche aegyptiaca* parasitism, as determined by performing quantitative RT-PCR. Clusters I, II, and III include genes that were up-regulated, constantly expressed, and down-regulated, respectively, at 1 d after *O. aegyptiaca* inoculation. The systemic expression of genes exhibiting greater than 8-fold up-regulation at either time point after *O. aegyptiaca* inoculation onto the *L. japonicus* roots was analysed. Systemic expression of genes exhibiting greater than 8-fold up-regulation in the root system was analysed in the leaves. Tile colours indicate the relative fold expression: green corresponds to less than 0.125-fold down-regulation; red, greater than 8-fold up-regulation; and yellow, constant expression (bottom-most panel) on comparing the inoculated and uninoculated samples (bottom-most panel).

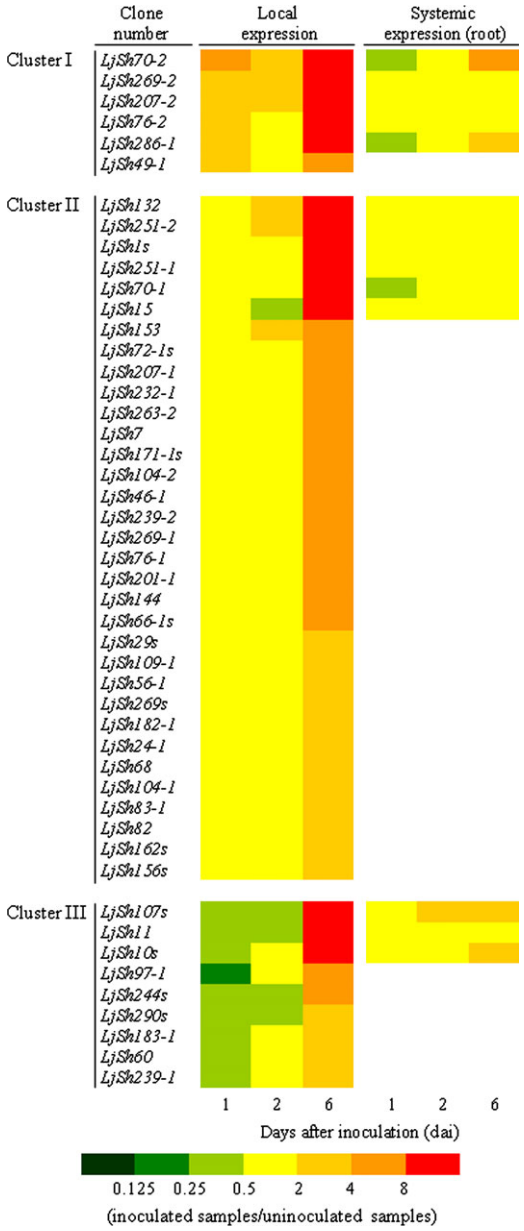

Fig. 3. Expression profiles of *Lotus japonicus* genes that were up-regulated in response to *Striga hermonthica* attachment as determined by performing quantitative RT-PCR. Clusters I, II, and III include genes that were up-regulated, constantly expressed, and down-regulated, respectively, at 1 d after *S. hermonthica* inoculation. The systemic expression of genes exhibiting greater than 8-fold up-regulation at either time point after *S. hermonthica* inoculation onto the *L. japonicus* roots was analysed. Tile colours indicate the relative fold expression: green corresponds to less than 0.125-fold down-regulation; red, greater than 8-fold up-regulation; and yellow, constant expression (bottom-most panel) on comparison of the inoculated and uninoculated samples (bottom-most panel).

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

Table 1. Genes showing up-regulated expression in the roots of *Lotus japonicus* after 10 d of *Orobanche aegyptiaca* inoculation

Clone	Homology (species; accession number)	*E*-value	Accession no.
Jasmonic acid biosynthesis			
LjOa25	Lipoxygenase (*Pisum sativum*; O24470)	3.8E-66	BB999902
LjOa83-1	Lipoxygenase (*Cicer arietinum*; Q9M3Z5)	2.0E-84	BB999909
LjOa74	Lipoxygenase mRNA (*Sesbania rostrata*; AJ309069)	2.0E-73	BB999926
Nodulation related			
LjOa198	Anti-H(O) lectin (*Lotus tetragonolobus*; P19664)	4.1E-112	BB999884
LjOa85s	Anti-H(O) lectin (*Lotus tetragonolobus*; P19664)	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	MtN19-like protein (*Pisum sativum*; AAU14999)	1.0E-11	BB999918
LjOa157-1	Actin-depolymerizing factor 2 (*Petunia×hybrida*; Q9FVI1)	3.8E-84	BB999921
Pathogenesis related			
LjOa9	Miraculin-like protein (*Solanum brevidens*; AAQ96377)	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*; O81926)	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*; Q39807)	1.6E-111	BB999899
LjOa214	Bowman-birk type proteinase inhibitor (*Amburana acreana*; P83284)	2.1E-97	BB999901
LjOa86	Serine proteinase inhibitor (*Medicago sativa*; Q40329)	3.8E-36	BB999912
LjOa227	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*; Q9M4D8)	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*; CAD89802)	6.0E-24	BB999916
LjOa33	Putative cytidine or deoxycytidylate 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 monooxygenase 1 (*Arabidopsis thaliana*; Q9LMA1)	4.0E-14	BB999907
LjOa40-1	Resistant specific protein-1(4) (*Vigna radiata*; Q8GSG3)	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 protein 75, isoform a (*Caenorhabditis elegans*; AAC48255)	0.34	BB999882
LjOa88	PGPS/D10 (*Petunia×hybrida*; Q9ZTM9)	1.4E-111	BB999911
LjOa24	UVI1 (*Pisum sativum*; Q9AUH7)	4.5E-08	BB999920
LjOa163	UVI1 (*Pisum sativum*; Q9AUH7)	1.2E-07	BB999914
LjOa51-1	Unknown protein (*Populus trichocarpa*; ABK94704)	0.001	BB999923
LjOa146	Unnamed protein (*Vitis vinifera*; CAO44822)	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	Pinoresinol-lariciresinol reductase homologue R5 mRNA (*Lotus japonicus*; AB265593)	1.8E-147	BB999951
LjSh29s	Cytochrome P450 mRNA (*Lotus japonicus*; AB025016)	1.4E-132	BB999933
Nodulation related			
LjSh1s	Small GTP-binding protein RAB11l mRNA (*Lotus japonicus*; Z73957)	3.0E-81	BB999929
Pathogenesis related			
LjSh70-2	Miraculin-like protein (*Solanum brevidens*; AAQ96377)	8.8E-17	BB999940
LjSh76-2	Class I chitinase (*Medicago sativa*; P94084)	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 (*Oryza sativa*; Q8GVW5)	2.0E-25	BB999938
LjSh251-2	Putative SKP1-like protein (*Oryza sativa*; Q8GVW5)	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*; Q9ZTF7)	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 acyltransferase (*Cucumis sativus*; Q08375)	3.1E-81	BB999954
LjSh104-1	Phosphoserine aminotransferase (*Arabidopsis thaliana*; Q8L7P0)	5.0E-14	BB999969
LjSh82	40S ribosomal protein S30 (*Arabidopsis thaliana*; P49689)	1.0E-09	BB999971
LjSh10s	Suspensor-specific protein (*Phaseolus coccineus*; AAK14318)	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)	3.8E-58	BB999976
Defence response			
LjSh49-1	Putative 1-aminocyclopropane-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 (*Solanum sogarandinum*; Q8H6E7)	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	BB999959
Cell-wall fortification			
LjSh104-2	Cinnamyl alcohol dehydrogenase-like protein gene (*Lotus corniculatus*; AY028929)	1.1E-147	BB999949
LjSh269-1	Putative cinnamyl alcohol dehydrogenase (*Oryza 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*; AF243362)	2.0E-52	BB999973
Other functions			
LjSh144	Snap25a (*Arabidopsis thaliana*; AAM62553)	2.7E-12	BB999963
LjSh66-1s	Kruppel like factor 4-like mRNA (*Danio rerio*; AM422104)	0.0002	BB999964
LjSh97-1	Serine/threonine-protein kinase tel1 (*Schizosaccharomyces pombe*; O74630)	0.18	BB999962
Unknown functions			
LjSh286-1	Coronin binding protein (*Dictyostelium discoideum*; O61085)	0.0011	BB999937
LjSh132	Uncharacterized Cys-rich domain (*Medicago truncatula*; ABD32291)	0.007	BB999957
LjSh15	Integral membrane family protein (*Arabidopsis thaliana*; NP_567472)	1.6E-46	BB999934
LjSh76-1	Uncharacterized Cys-rich domain (*Medicago truncatula*; ABD32291)	7.0E-14	BB999960
LjSh107s	UPF0497 membrane protein (*Arabidopsis thaliana*; Q9SQU2)	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).

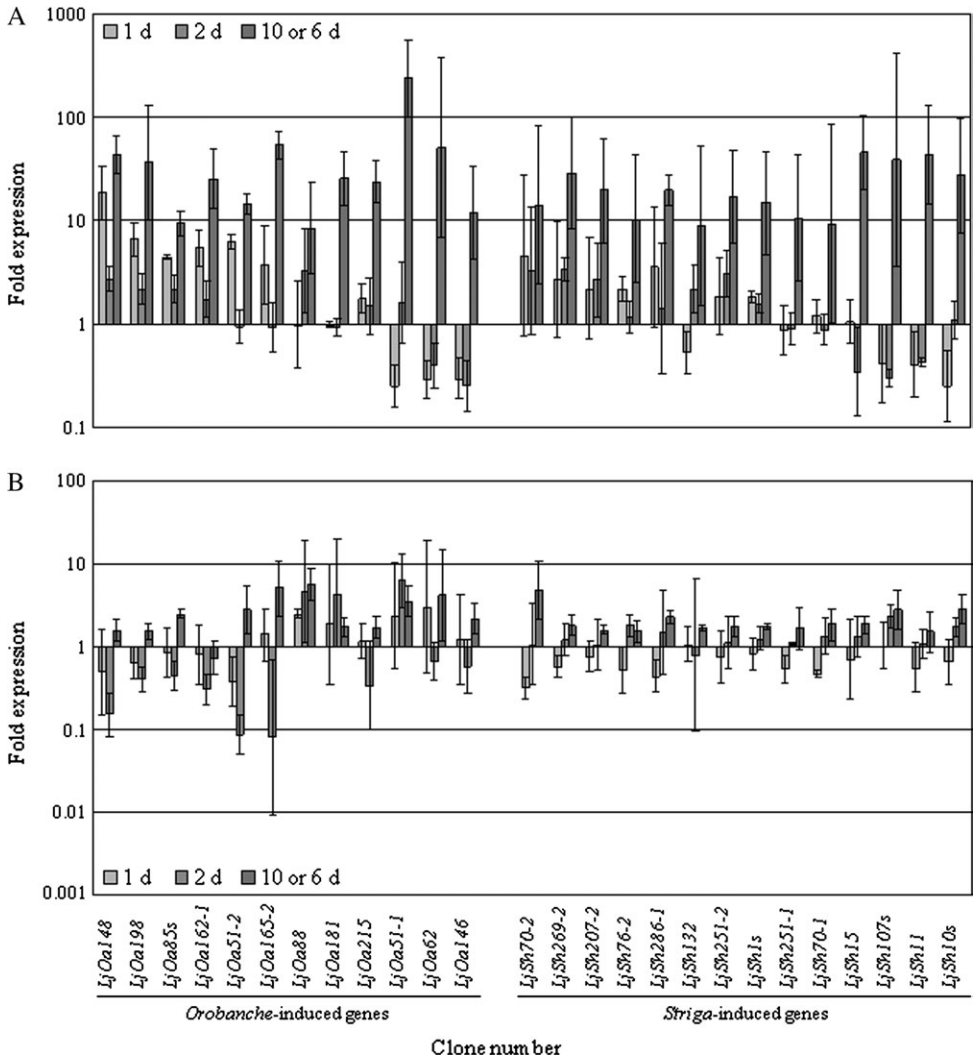

Fig. 4. Local (A) and systemic (B) expression of the 12 *Orobanche*-induced genes and the 14 *Striga*-induced genes in *Lotus japonicus* roots. The *x*-axis indicates the clone number and the days after inoculation of each gene. The *y*-axis indicates fold expression induction (log scale). The error bars represent the SD of the inductions.

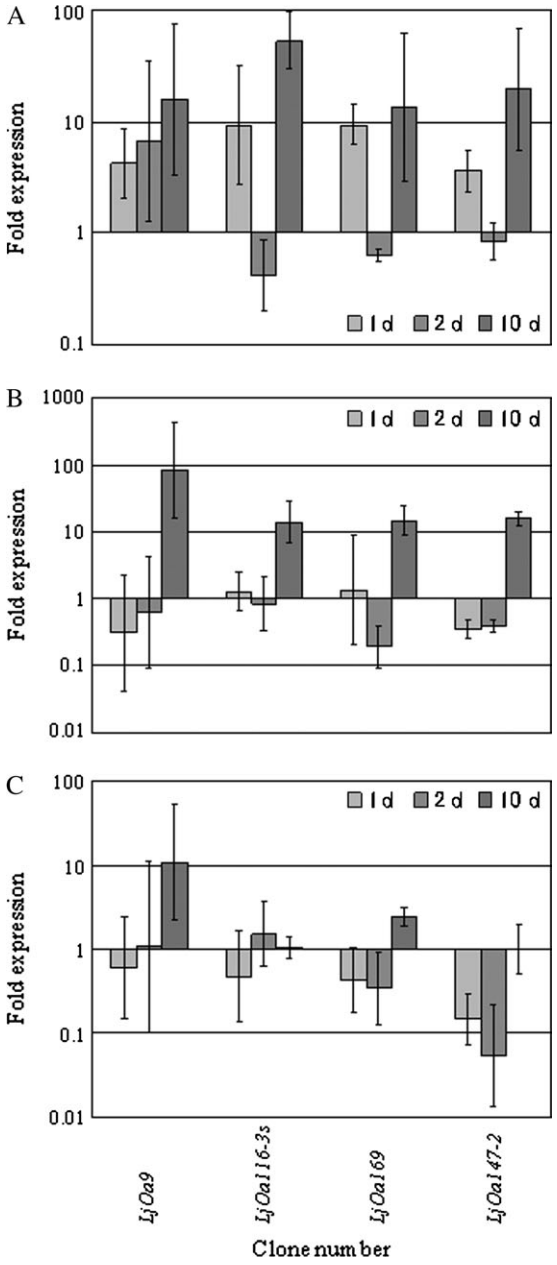

Fig. 5. Local expression (A) and systemic expression (B) in *Lotus japonicus* roots, and systemic expression (C) in *L. japonicus* leaves of the four *Orobanche*-induced genes. The *x*-axis indicates the clone number and the days after inoculation of each gene. The *y*-axis indicates fold expression induction (log scale). The error bars represent the SD of the inductions.

Discussion

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.* (2003*b*), 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.*, 2003*a*, *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 bifrorus*, 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 *LjOa95*, 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. hermonthica*, 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 *LjOa9*, 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. hermonthica* 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. hermonthica* 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.

Acknowledgements

The authors thank Ms Ayaka Hamasaki and Ms Miho Sugo for their technical assistance in various aspects of the study. This work was supported, in part, by a Grant-in-Aid for Scientific Research (B) (Nos 17405027 and 20405026), by a grant from the AA Science Platform Program of the Japan Society for the Promotion of Science, and by a grant from the Sumitomo Foundation to YS.

References

Berner DK, Kling JG, Singh BB. 1995. *Striga* research and control. *Plant Disease* **79,** 652–660.

Bonde MR, Millar RL, Ingham JL. 1973. Induction and identification of sativan and vestitol as two phytoalexins from *Lotus corniculatus*. *Phytochemistry* **12,** 2957–2959.

Diatchenko L, Lau YFC, Campbell AP, *et al*. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences, USA* **93,** 6025–6030.

Die JV, Dita MA, Krajinski F, González-Verdejo CI, Rubiales D, Moreno MT, Román B. 2007. Identification by suppression subtractive hybridization and expression analysis of *Medicago truncatula* putative defence genes in response to *Orobanche crenata* parasitization. *Physiological and Molecular Plant Pathology* **70,** 49–59.

Goldwasser Y, Kleifeld Y. 2004. Recent approaches to *Orobanche* management. In: Inderjit, ed. *Weed biology and management*. Dordrecht, the Netherlands: Kluwer Academic Publishers, 439–466.

Gowda BS, Riopel JL, Timko MP. 1999. *NRSA-1*: a resistance gene homolog expressed in roots of non-host plants following parasitism by *Striga asiatica* (witchweed). *The Plant Journal* **20,** 217–230.

Graham MA, Silverstein KAT, Cannon SB, VandenBosch KA. 2004. Computational identification and characterization of novel genes from legumes. *Plant Physiology* **135,** 1079–1197.

Griffitts AA, Cramer CL, Westwood JH. 2004. Host gene expression in response to Egyptian broomrape (*Orobanche aegyptiaca*). *Weed Science* **52,** 697–703.

Hiraoka Y, Sugimoto Y. 2008. Molecular responses of sorghum to purple witchweed (*Striga hermonthica*) parasitism. *Weed Science* **56,** 356–363.

Joel DM, Portnoy VH. 1998. The angiospermous root parasite *Orobanche* L. (Orobanchaceae) induces expression of a pathogenesis related (PR) gene in susceptible tobacco roots. *Annals of Botany* **81,** 779–781.

Kalsi G, Etzler ME. 2000. Localization of a nod factor–binding protein in legume roots and factors influencing its distribution and expression. *Plant Physiology* **124,** 1039–1048.

Kosslak RM, Bohlool BB. 1984. Suppression of nodule development of one side of a split-root system of soybeans caused by prior inoculation of the other side. *Plant Physiology* **75,** 125–130.

Kubo M, Ueda H, Park P, Kawaguchi M, Sugimoto Y. 2008. Reactions of *Lotus japonicus* ecotypes and mutants to root parasitic plants. *Journal of Plant Physiology* 10.1016/j.jplph.2008.06.007.

Liechti R, Farmer EE. 2002. The jasmonate pathway. *Science* **296,** 1649–1650.

Lozano-Baena MD, Prats E, Moreno MT, Rubiales D, Pérez–3de–Luque A. 2007. *Medicago truncatula* as a model for non-host resistance in legume–parasitic plant interactions. *Plant Physiology* **145,** 437–449.

Mason HS, Mullet JE. 1990. Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding, and jasmonic acid. *The Plant Cell* **2,** 569–579.

Mohamed A, Ellicott A, Housley TL, Ejeta G. 2003. Hypersensitive response to *Striga* infection in *Sorghum*. *Crop Science* **43,** 1320–1324.

Musselman LJ, Matteson PC, Fortune S. 1983. Potential pollen vectors of *Striga hermonthica* (Scrophulariaceae) in West Africa. *Annals of Botany* **51,** 859–862.

Roberts NJ, Brigham J, Wu B, Murphy JB, Volpin H, Phillips DA, Etzler ME. 1999. A nod factor-binding lectin is a member of a distinct class of apyrases that may be unique to the legumes. *Molecular and General Genetics* **262,** 261–267.

Rubiales D. 2003. Parasitic plants, wild relatives and the nature of resistance. *New Phytologist* **160,** 459–461.

Shimada N, Sato S, Akashi T, Nakamura Y, Tabata S, Ayabe S, Aoki T. 2007. Genome-wide analyses of the structural gene families involved in the legume-specific 5-deoxyisoflavonoid biosynthesis of *Lotus japonicus*. *DNA Research* **14,** 25–36.

Sugimoto Y, Ali AM, Yabuta S, Kinoshita H, Inanaga S, Itai A. 2003. Germination strategy of *Striga hermonthica* involves regulation of ethylene biosynthesis. *Physiologia Plantarum* **119,** 137–145.

VandenBosch KA, Stacey G. 2003. Summaries of legume genomics projects from around the globe. community resources for crops and models. *Plant Physiology* **131,** 840–865.

Vieira-Dos-Santos C, Delavault P, Letousey P, Thalouarn P. 2003*a*. Defence gene expression analysis of *Arabidopsis thaliana* parasitized by *Orobanche ramosa*. *Phytopathology* **93,** 451–457.

Vieira-Dos-Santos C, Delavault P, Letousey P, Thalouarn P. 2003*b*. Identification by suppression subtractive hybridization and expression analysis of *Arabidopsis thaliana* putative defence genes during *Orobanche ramosa* infection. *Physiological and Molecular Plant Pathology* **62,** 297–303.

Westwood JH, Yu X, Foy CL, Cramer CL. 1998. Expression of a defence-related 3-hydroxy-3-methylglutaryl CoA reductase gene in response to parasitization by *Orobanche* spp. *Molecular Plant–Microbe Interactions* **11,** 530–536.

Wilson RC, Long F, Maruoka EM, Cooper JB. 1994. A new proline-rich early nodulin from *Medicago truncatula* is highly expressed in nodule meristematic cells. *The Plant Cell* **6,** 1265–1275.