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The Endosymbiosis-Induced Genes *ENOD40* and *CCS52a* Are Involved in Endoparasitic-Nematode Interactions in *Medicago truncatula*

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Plants associate with a wide range of mutualistic and parasitic biotrophic organisms. Here, we investigated whether beneficial plant symbionts and biotrophic pathogens induce distinct or overlapping regulatory pathways in *Medicago truncatula*. The symbiosis between *Sinorhizobium meliloti* and this plant results in the formation of nitrogen-fixing root nodules requiring the activation of specific genes in the host plant. We studied expression patterns of nodule-expressed genes after infection with the root-knot nematode *Meloidogyne incognita*. Two regulators induced during nodule organogenesis, the early nodulin gene *ENOD40* involved in primordium formation and the cell cycle gene *CCS52a* required for cell differentiation and endoreduplication, are expressed in galls of the host plant. Expression analysis of promoter-*uidA* fusions indicates an accumulation of *CCS52a* transcripts in giant cells undergoing endoreduplication, while *ENOD40* expression is localized in surrounding cell layers. Transgenic plants overexpressing *ENOD40* show a significantly higher number of galls. In addition, out of the 192 nodule-expressed genes tested, 38 genes were upregulated in nodules at least threefold compared with control roots, but only two genes, nodulin 26 and cyclin D3, were found to be induced in galls. Taken together, these results suggest that certain events, such as endoreduplication, cell-to-cell communication with vascular tissues, or water transport, might be common between giant cell formation and nodule development.

During evolution, plants have engaged associations with a wide range of mutualistic and parasitic biotrophic organisms, ranging from bacteria to nematodes. The common feature of biotrophic interactions is that the invading organisms penetrate into the plant cell where they are separated from the cytoplasm by a plant membrane. Therefore, it is quite conceivable that these interactions might have evolved certain common core components affecting cellular functions, such as cell-wall reorganization, membrane synthesis, metabolite fluxes, or cytoskeleton rearrangements (Parniske 2000). In spite of the fact that the development and physiology of biotrophic interactions are specific and significantly different from each other (depending on the type of microorganism), those interactions occurring between plants and endoparasitic nematodes and the legume-*Rhizobium* symbioses are among the most elaborate.

Rhizobium-induced nodulation is highly specific and almost restricted to leguminous plants. In these interactions, Nod factors secreted by rhizobia play a central role as external mitogenic signals that induce cell division in the root cortex and the expression of several genes, including those related to cell cycle activation and the early nodulin gene *ENOD40* (Foucher and Kondorosi 2000). Differentiation of the nodule primordium starts by division arrest, and these cells undergo multiple rounds of endocycles and a gradual increase in cell volume. These endoreduplicating and growing cells can only be invaded by rhizobia and develop to symbiotic nitrogen-fixing cells. Among the genes shown to be involved in nodulation, *ENOD40* has been studied both as a marker for the early rhizobial Nod factor-induced responses in plants and as a regulator of primordium formation (Charon et al. 1999; Fang and Hirsch 1998; Yang et al. 1993). The CCS52 protein is an APC activator involved in mitotic cyclin degradation and in regulation of endoreduplication in root nodule organogenesis (Cebolla et al. 1999). Two *CCS52* genes have been identified in the model legume *Medicago truncatula* (*CCS52a*, Cebolla et al. 1999; and *CCS52b*, E. Kondorosi, *unpublished data*). However, *ENOD40* and *CCS52* are not exclusively associated with the nodulation process (Foucher and Kondorosi 2000).

Genetic and molecular approaches have identified plant genes involved in nodulation. Several of them are common to mycorrhizae and nodule formation, since they are induced during both symbiotic interactions (Albrecht et al. 1999), notably early nodulin genes as *ENOD40* (van Rhijn et al. 1997). Indeed, the most convincing evidence that the infection processes used by both microsymbionts involve common steps is that a large proportion of the nodulation-resistant mutants were also completely resistant to arbuscular endomycorrhiza fungi (Gianinazzi-Pearson 1996).

The root-knot nematode (RKN) *Meloidogyne* spp. are able to induce nematode feeding sites (NFS) in several thousand host species, in contrast to the restricted host range of rhizobia. The NFS comprises a group of giant cells that develop from root cells close to the xylem (Williamson and Hussey 1996). In response to the parasite (probably due to salivary secretions), these cells undergo nuclear division without cytokinesis, cycles of endoreduplication, and alterations of their cell wall and cytoplasm content resulting in multinucleated and hypertrophied cells (Wiggers et al. 1990). Simultaneously, cells in the neighboring tissues start to divide, and this proliferation gives

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rise to the formation of a typical root knot or gall. These cytological observations indicate that root-knot nematodes also induce cell cycle activation and reprogramming of host cellular fate (de Almeida Engler et al. 1999).

In contrast to the *Rhizobium*-legume symbioses, little is known about the plant genetic requirements for pathogenic interactions. Few inducible genes have been isolated and well characterized during susceptible infections. This is the case for the *fis-1* gene encoding a putative aldehyde dehydrogenase, which is specifically induced in flax leaves during interaction with the flax rust fungus *Melampsora lini* (Roberts and Pryor 1995), and the *RPE* gene encoding a ribulose-5-phosphate-3-epimerase, which has been shown to be essential for the NFS formation during infection with the RKN *Meloidogyne incognita* (Favery et al. 1998).

In order to reveal common regulatory pathways between root symbioses and nematode interactions, we studied the expression pattern of *ENOD40* and *CCS52* nodule genes during gall formation in *M. truncatula*, using transgenic plants carrying promoter *uidA* fusions. In addition, the consequences of overexpression of *ENOD40* in *M. truncatula* plants on infection by the RKN *Meloidogyne incognita* was analyzed. Finally, expression profiling of 192 nodule-expressed genes was carried out for these interactions. This represents the most exhaustive survey carried out so far. Our results indicate that certain cellular events dealing with endoreduplication, cell cycle regulation, and cell-to-cell communication processes seem to be involved in both biotrophic interactions.

RESULTS

MtENOD40 and *MtCCS52a* expressions in NFS.

To examine gene regulation during plant-nematode interaction, we first analyzed the expression patterns of *ENOD40* and *CCS52a* genes in transgenic plants carrying the promoters of these genes fused to the β-glucuronidase reporter gene. During root development *ENOD40* is weakly expressed in the vascular cylinder, whereas CCS52 is expressed in the root apical meristem and lateral root initiation sites (data not shown). After *Meloidogyne incognita* nematode infection, both genes were expressed at 7 days postinfection (dpi) in galls (Fig. 1A and B). Time course experiments showed that both genes were induced in young galls 3 dpi, i.e., 24 to 48 h after giant cell initiation, and persisted in 15 dpi galls. No GUS staining was detected at the penetration site (in the elongation zone) or during nematode migration.

To determine the precise localization of GUS expression, thin sections of NFS were made. Cross sections of 7-day-old galls revealed different patterns of *ENOD40* and *CCS52a* expression. The *ENOD40* gene was induced in different gall tissues: in the vascular system, in those cells that are in direct contact with the pathogen or surrounding giant cells, and in the gall cortex at a certain distance from the nematode (Fig. 1C). However, no GUS expression was found inside giant cells. On the contrary, a high induction of the *CCS52a* gene was observed in giant cells, consistently with the function of this gene in endoreduplication, and also in surrounding cells in which differentiation occurs (Fig. 1D).

Increased gall formation in *ENOD40*-overexpressing plants.

The increased *ENOD40* promoter activity in galls suggested that this gene, similarly to its action in nodule development, might play a role in nematode-induced cell proliferation and, thereby, gall formation. Therefore, we analyzed whether overexpression of *ENOD40* in two independent transgenic lines (Se40-3 and Se40-10 lines) (Charon et al. 1997) affect gall formation induced by the RKN *Meloidogyne incognita.* Microscopic analysis of galls indicated no modification in the structure of giant cells in these two lines compared with wild-type *M. truncatula* (data not shown). However, the number of galls was significantly increased at 7 dpi in Se40 plants compared with

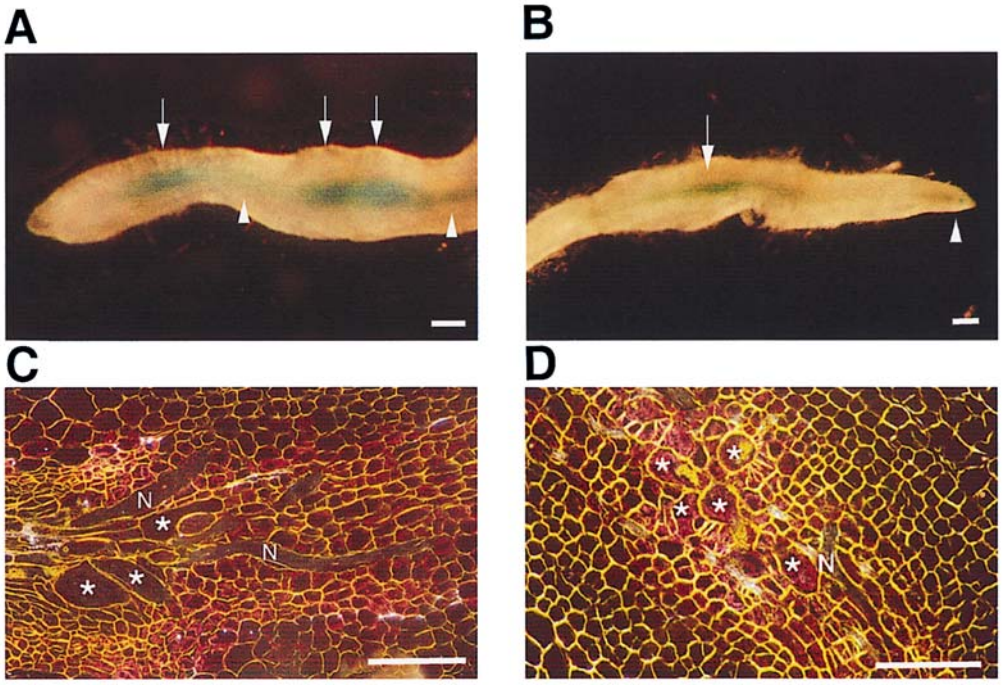


Fig. 1. GUS expression in galls induced by *Meloidogyne incognita* in *Medicago trunculata* plants transformed with constructs carrying *ENOD40* and *CCS52a* promoter-*GUS* fusions. **A** and **B,** Localized GUS expression in root gall (arrows) 7 days after infection in **A,** *ENOD40* and **B,** *CCS52a* promoter-*uidA* fusion plants. Arrowheads indicate background GUS expression unrelated to nematode infection and observed also in uninfected roots. **C** and **D,** Cross sections of galls examined under dark field light. **C,** *ENOD40* promoter-*uidA* fusion: GUS expression (pink precipitate) is observed only in cells surrounding the giant cells (*) and the nematode (N). **D,** *CCS52a* promoter-*uidA* fusion: GUS expression in giant cells and surrounding cells. Bar = 100 μm.

wild-type control plants or with transgenic *M. truncatula* carrying the *ENOD40* promoter-GUS fusion. Since no significant differences in nematode infection were observed between the two Se-40 lines, data were pooled together (Fig. 2). Time course experiments were then carried out to show that overexpression of *ENOD40* resulted in an increased number of galls at 7 and 15 dpi, compared with control lines ($P < 0.01$).

Expression profiles of genes induced in galls and nodules.

Involvement of *ENOD40* and *CCS52a* in both nodule and gall formation suggested that other genes, or perhaps even regulatory pathways, might be common to these interactions. To compare changes in gene expression profiles during nodule and gall development, cDNA macroarrays were assembled with 192 expressed sequence tags (EST) from a young-nodule cDNA library (discussed below). Three sets of filters were hybridized with ^{33}P-labeled cDNAs synthesized from total RNA samples extracted from control uninfected roots, nematode galls (7 and 14 dpi), and root nodules (20 dpi). In these macroarrays, genes coding for known nodulins, putative regulatory genes, general housekeeping and metabolic genes, as well as several EST corresponding to unknown functions (no homology in databases) were included.

Three weeks after inoculation of *M. truncatula* with *S. meliloti*, a marked change in the global expression pattern of the tested genes was observed (Fig. 3A). In the case of galls induced by the nematodes, few changes were detected in the expression pattern of these genes. Genes were regarded as *Rhizobium*- or nematode-induced when the change in mRNA abundance compared with that in untreated roots was greater than 3. Triplicate experiments were repeated twice, and reproducible results obtained for 133 genes were analyzed. Based on this criterion, mRNA levels for 38 genes were upregulated in nodules. These genes correspond to different gene classes: 22 correspond to known nodulin or metabolic genes (e.g., *ENOD40*, *ENOD20*, *ENOD27*, *ENOD22*, leghemoglobins, carbonic anhydrase, MRP-like ABC transporter, chitinase), 12 to new nodulins with no homology in databases, and 4 to putative regulatory genes (one transcription factor scarecrow-like, two protein kinase homologs, and one calmodulin-binding protein). In nematode-infected tissues, three and five genes were upregulated after 7 and 14 dpi, respectively. Among them, nodulin 26 (*NOD26*) and cyclin D3 (*CYCD3*) were the only genes activated both times during the nematode infection kinetics. Using macroarray analysis, similar levels of expression of the *ENOD40* and *CCS52a* genes were detectable in galls and roots, whereas *ENOD40* was induced at high levels in nodule cells. Gene induction in nodules was high, up to 531-fold (e.g., for the nodulin 25 precursor), whereas in galls, the most upregulated gene, *NOD26*, displayed only a 5.4-fold increase in its expression. This gene, encoding an aquaporin (Dean et al. 1999), showed a 44-fold induction in nodules. The regulation of the other gene induced in galls, the D-type cyclin *CYCD3*, was different, since it was activated only in nematode-infected tissues. Induced expression of these two new nematode-induced genes was confirmed using semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR), and the induction levels are comparable to those obtained with the macroarrays (Fig. 3B and C).

DISCUSSION

In this work, we analyzed the expression patterns of nodule-expressed genes in the interaction between *M. truncatula* and the RKN *Meloidogyne incognita* to see whether mutualistic and parasitic biotrophic organisms operate through overlapping regulatory pathways. Our results show that the early nodulin *ENOD40* and the cell cycle *CCS52a* genes are locally induced in galls of the host plant *M. truncatula* by the nematode *Meloidogyne incognita*. Expression patterns of these genes were different in NFS, consistent with their different functions. In galls, *ENOD40* expression was detected in cells surrounding the giant cells but not within the giant cells. We could not detect *ENOD40* expression using in situ hybridization in galls (data not shown). However, a previous experiment done by in situ RT-PCR showed *ENOD40* upregulation in giant cells and their surrounding cells (Koltai et al. 2001). The detection threshold of these techniques could account for these differences in *ENOD40* localization in galls. The role of this gene during the plant-nematode interaction has also been analyzed using plants overexpressing *ENOD40*. The constitutive overexpression of this gene resulted in significant increase of NFS induction, and no significant differences in root development were detected at these timepoints when large root systems are present in both types of plants. In the plant-*Rhizobium* interaction, this gene was expressed first in the pericycle cells opposite to the protoxylem poles within a few hours after inoculation with *Rhizobia* cells and markedly before the first cell divisions occurred in the root cortex (Yang et al. 1993; Mathesius et al. 2000). In plants overexpressing *ENOD40*, increased initiation of nodule primordia was observed at early timepoints, which was accompanied by a proliferation response close to the root tip (Charon et al. 1999). Therefore, it has been proposed that *ENOD40* induction is a limiting step in primordium formation, sensitizing the cortical cells for division. ENOD40 could play a role in transport of compounds, such as carbohydrates, into the cortex from pericycle tissues to allow proper organization of the primordium (Charon et al. 1999; Kouchi et al. 1999). During the plant-nematode interaction, gall formation also takes place close to the root tip and results from the induction of giant cells and dividing cortical cells around the nematode, which is consistent with the proposed role of *ENOD40* in cell-to-cell communication processes between vascular and cortical root tissues.

The *CCS52a* gene was also found to be upregulated during gall formation. During nodule development, *CCS52a* was expressed in the persistent meristem and nodule zone II, in which cell division is arrested and cells undergo multiple rounds of endoreduplication cycles leading to development of large symbiotic cells. CCS52a is a cell cycle regulator that links cell proliferation to cell differentiation and promotes endoreduplication and cell enlargement (Cebolla et al. 1999). During gall formation, we demonstrated that *CCS52a* was expressed in NFS within the giant cells and their surrounding cells. In line with these results, DNA synthesis endocycles and progression until the G2 phase or mitosis occur in these cells and were shown to be essential for gall establishment (de Almeida Engler et al. 1999). This supports the idea that this gene has a general role in organ developmental processes (Cebolla et al. 1999). Therefore, our data on *ENOD40* and *CCS52a* expression suggest that there are common elements in the development of nematode galls and nitrogen-fixing nodules. However, these genes are not exclusively associated to nodulation, since they are expressed in several other cell types and nonlegume homologs exist for both genes (Kouchi et al. 1999; Cebolla et al. 1999).

The analysis of expression profiles of a larger number of *M. truncatula* genes in response to infection by *Rhizobium* spp. and the RKN *Meloidogyne incognita* provides a base to identify specific genes involved in both interactions. In this study, we examined changes in the expression patterns of 192 selected genes (including cell cycle genes and putative elements of signal transduction pathways) by cDNA macroarray analysis after infection with the RKN *Meloidogyne incognita* and *S. meliloti*. Our data revealed that only a few genes are signifi-

cantly upregulated in both interactions. NOD26 is a member of the aquaporin channel family, which confers water and glycerol transport capacities to the symbiosome membrane and plays a role in osmoregulation during legume-*Rhizobium* (Dean et al. 1999) and legume-endomycorrhizal symbioses (Albrecht et al. 1999). During NFS formation, nematodes induce a dramatic alteration in cell development, leading to production of giant cells. A typical trait of these structures is their high metabolic activity (Favery et al. 1998). The upregulation of *NOD26* in NFS together with the activation of the water-channel *TOB-RB7* in tobacco giant cells (Opperman et al 1994) indicate a concerted activation of genes dealing with turgor pressure and osmotic potential of the giant cells.

The D-type cyclin gene *CYCD3* is induced in galls 7 and 15 dpi. This gene is induced in the G1 phase of the cell cycle (Dahl et al. 1995). During gall development, expression patterns of other key regulatory genes, such as the mitotic cyclin *CYCB1;1*, *CDC2bAt*, and *CYCA2;1*, has been reported in *Arabidopsis* (de Almeida Engler et al. 1999). The activation of *CYCD3* suggests that the D-type cyclins also play a key role in the response of root cells for nematodes to proceed into the cell cycle (at least until late G2) or to initiate endoreduplication, or both. During nodule development, we were not able to detect *CYCD3* upregulation in the macroarray experiment. However, expression analysis of the *Medicago sativa CYCD3* revealed a short and transient induction in early stages of *S. meliloti* infection and also in later stages of nodule development (Foucher and Kondorosi 2000).

The results of expression data observed by this macroarray analysis were in accordance with those obtained by RT-PCR for *NOD26* and *CYCD3*. However the macroarray analysis did not show induction of *CYCD3* in nodules or *ENOD40* and *CCS52a* in NFS. This was also confirmed for the latter two using RT-PCR. These differences in the results obtained with macroarrays or other detection methods (transgenic plants, in situ expression) may be explained by a transient and early activation of these genes during NFS formation and nodule development or by the fact that their expression was very localized and hidden by the background expression detected using large samples from uninfected root tissues, as required for cDNA labeling. Alternatively, there may also exist differences between steady-state transcript levels (measured by cDNA microarrays or in situ hybridization) and promoter activation (monitored through reporter gene expression).

Within the scope of addressing the question of common signal-transduction pathways between root symbioses and endopathogenic interactions, our data suggest that very specific signals from biotrophic organisms trigger signaling pathways in the plant host. These data may be linked to previous results indicating that RKN infection remains essentially unaffected in plants expressing systemic acquired resistance against bacterial and fungal pathogens. The signals involved likely differ substantially between RKN and rhizobia since, out of 37 genes specific for nodule development, very few are activated in NFS. However, for certain cellular processes (i.e., genes involved in cell cycle or phytohormone responses), signaling pathways are likely to be common between RKN and rhizobia and probably also for other intimate biotrophs, such as mycorrhizae. The recent data indicating that two transcriptional regulators, PHAN and KNOX, required for the establishment of meristems are expressed in nodules and in giant cells supports this hypothesis (Koltai et al. 2001). Among other plant functions (which may be discovered using larger arrays), our results indicate that certain of those dealing with endoreduplication, cell cycle regulation, cell to cell communication, and water transport are shared by the complex developmental processes of nodule organogenesis and gall formation.

MATERIALS AND METHODS

Plant materials and infection with nematodes.

Plants were grown in a greenhouse on sand at 20°C under a 16-h-light and 8-h-dark regime. Seeds were surface sterilized as described by Charon and associates (1997). Plants (3 weeks old) were inoculated with 3,000 *Meloidogyne incognita* second stage juveniles (J2). The inoculated plants were carefully harvested from 3 to 15 days after inoculation, and NFS were counted by visual scoring. No differences in root growth or length of these plants were observed. For testing *ENOD40* and *CCS52a* promoter activity, β-glucuronidase activity (GUS staining) was followed in time course experiments according to Favery and associates (1998).

M. truncatula Se40-3 and Se40-10 lines overexpressing the early nodulin gene *ENOD40* have been described and characterized (Charon et al. 1997, 1999). For expression analysis of *ENOD40*, a 1.2-kb fragment of the *MtENOD40* promoter was introduced into a pPGTV-bar vector (Becker et al. 1992) containing a *uidA* reporter gene. This construct was transferred into *M. truncatula* as described (Charon et al., 1997) and T_2 descendants, exhibiting GUS activity in nodules as expected (Fang and Hirsch et al. 1998), were used to monitor *ENOD40* expression following nematode infection. Similarly, a 2.4-kb fragment carrying the *MtCCS52a* promoter fused to a promoterless *uidA* gene in the binary vector pPR97 (Szabados et al. 1995) was transformed into *M. truncatula*. GUS staining was used to study *MtCCS52a* promoter activity on the T_2 population. Detailed characterization of these plants will be published elsewhere (J. M. Vinardell, *unpublished data*).

Histochemical localization of GUS activity and microscopic analyses.

Galls were dissected from GUS-stained plants, fixed in 1% glutaraldehyde and 4% formaldehyde in 50 mM sodium phosphate buffer, pH 7.2, dehydrated, and embedded in Technovit 7100 (Hereaus-Kulzer, Wehrheim, Germany), as instructed by the manufacturer. Sections (4 μm) were made on a JUNG microtome, stained with 0.05% ruthenium red in water at room temperature for 8 min, and mounted in DPX mountant (BDH Laboratory Supplies, Poole, England). Sections were observed

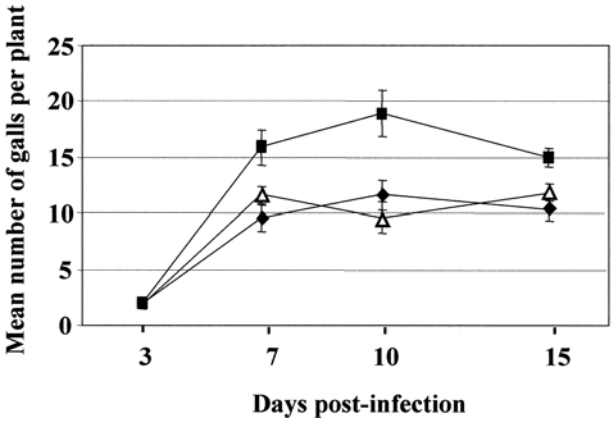


Fig. 2. Progression of nematode infection in *ENOD40*-overexpressing *Medicago truncatula* lines and control plants. The number of galls per plant was determined 3 to 15 days postinfection on plants overexpressing *ENOD40* (Se40, squares). Since no significant differences in nematode infection were observed between Se40-3 and Se40-10 lines, data were pooled together. Untransformed *M. truncatula* (MT0, lozenges) and plants transformed with *ENOD40* promoter-*uidA* fusion (ProE40, open triangles) were used as controls. Values represent the means ± standard error obtained on 40 plants inoculated with *Meloidogyne incognita* second stage juveniles in two independent experiments.

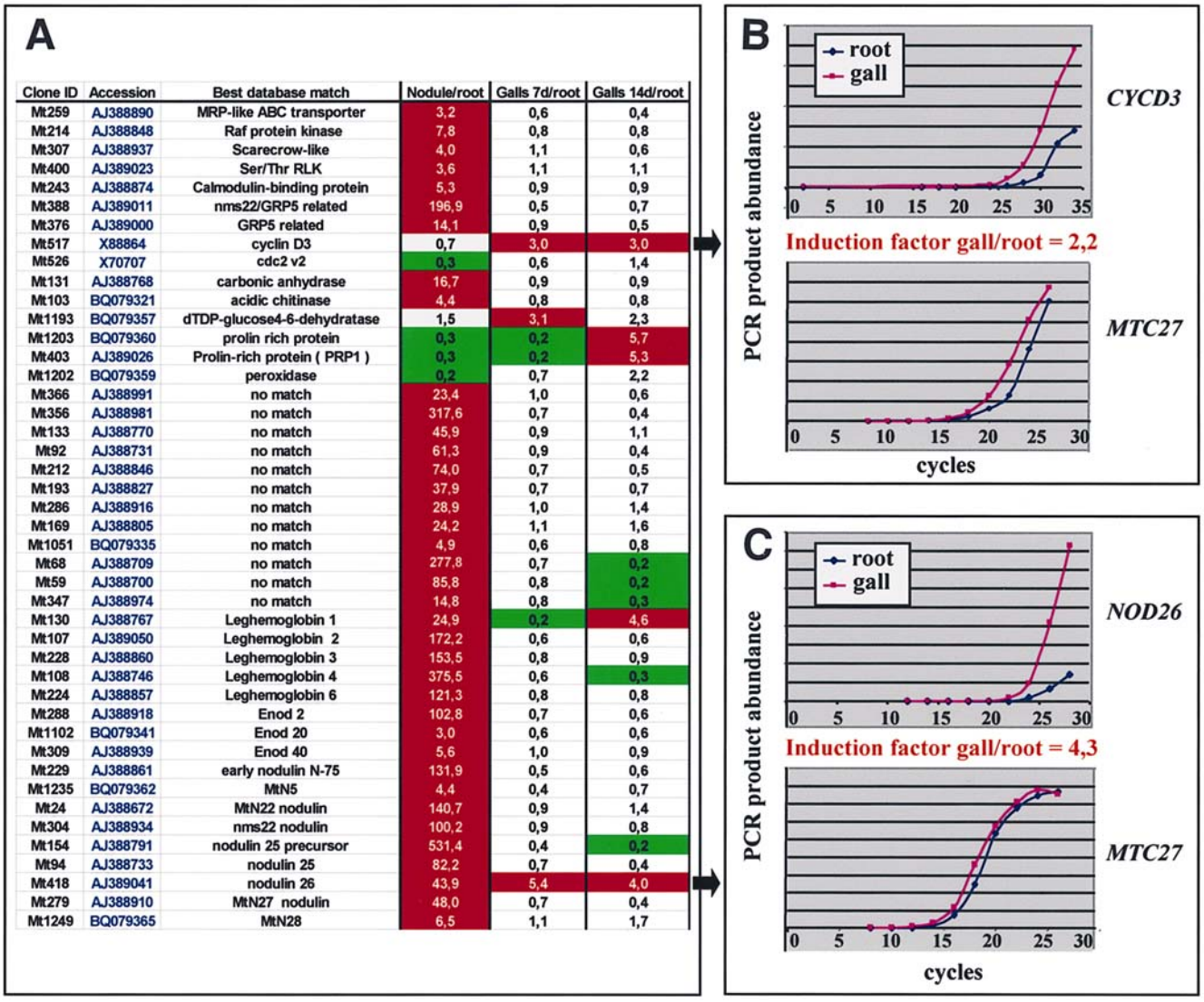
through a Zeiss Axioplan 2 microscope with dark field illumination to increase the sensitivity of the detection.

Macroarray and RT-PCR analysis.

Galls were excised one and two weeks after nematode infection. Nodules were yielded 20 days after inoculation of *M. truncatula* roots with *S. meliloti*. As a control tissue, nonmeristematic root fragments were obtained from uninfected plants. Immediately after excision, plant material was frozen in liquid nitrogen. Total RNA was prepared as described (Sambrook et al. 1989) and further purified with the RNeasy kit (Qiagen S.A., Courtaboeuf, France).

For semiquantitative RT-PCR, cDNA was prepared from 20 ng of polyA+ RNA with Superscript reverse transcriptase (Gibco-BRL/Life Technologies, Gaithersburg, MD, U.S.A.). The expression of the *MTC27* gene *(M. truncatula* homologue of *M. sativa MSC27* gene) was used for normalization of expression results. Gene specific-fragments were amplified by PCR using the following primers: nodulin 26, 5′ GTG-TAC-CTA-ACA-GTG-TTG-TAG-ACC-CA; nodulin 26, 3′ GCT-CCT-TTG-TCT-CTG-TCC-CTT-TCT-TGC; *CYCD3*, 5′ GAC-TGA-ACA-AAG-AGG-AAA-AGA-GAG-AGC; *CYCD3*, 3′ GCT-TCA-CGA-CGA-GGT-TGA-GTG-AGT-GAG; *MTC27*, 5′ GGA-GGT-TGA-GGG-AAA-GTG-G; *MTC27*, 3′ CAC-CAA-CAA-AGA-ATT-GAA-GG and using the amplification protocol: 94°C for 1 min.; followed by cycles of 94°C for 40 s, 55°C for 40 s, 72°C for 1 min, and finishing with 72°C for 5 min. Cycles were repeated in a series of every two cycles: from 8 to 26 for *MTC27*, from 12 to 30 for *NOD26*, and from 16 to 34 for *CYCD3*, in order to estimate the relative template concentration. PCR products were separated on a 1% agarose gel, and quantification was done on a Southern blot, hybridized with specific probes, and analyzed with a STORM Phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Sigmoid curves correspond in their exponential part to the formula: $y = t\,2^x$ where y is the amount of PCR product; t, the initial amount of template; and x, the number of PCR cycles. Log transformation of these curves ($\ln y = \ln t + x \ln 2$) was used to estimate template amounts (t) for the different genes and samples, thus allowing calculation of induction factors normalized through the t_{MTC27} values.

Macroarrays containing 192 EST from a nodule cDNA library (Györgyey et al. 2000) were made with a BIOMEK 2000



Clone ID	Accession	Best database match	Nodule/root	Galls 7d/root	Galls 14d/root
Mt259	AJ388890	MRP-like ABC transporter	3,2	0,6	0,4
Mt214	AJ388848	Raf protein kinase	7,8	0,8	0,8
Mt307	AJ388937	Scarecrow-like	4,0	1,1	0,6
Mt400	AJ389023	Ser/Thr RLK	3,6	1,1	1,1
Mt243	AJ388874	Calmodulin-binding protein	5,3	0,9	0,9
Mt388	AJ389011	nms22/GRP5 related	196,9	0,5	0,7
Mt376	AJ389000	GRP5 related	14,1	0,9	0,5
Mt517	X88864	cyclin D3	0,7	3,0	3,0
Mt526	X70707	cdc2 v2	0,3	0,6	1,4
Mt131	AJ388768	carbonic anhydrase	16,7	0,9	0,9
Mt103	BQ079321	acidic chitinase	4,4	0,8	0,8
Mt1193	BQ079357	dTDP-glucose4-6-dehydratase	1,5	3,1	2,3
Mt1203	BQ079360	prolin rich protein	0,3	0,2	5,7
Mt403	AJ389026	Prolin-rich protein (PRP1)	0,3	0,2	5,3
Mt1202	BQ079359	peroxidase	0,2	0,7	2,2
Mt366	AJ388991	no match	23,4	1,0	0,6
Mt356	AJ388981	no match	317,6	0,7	0,4
Mt133	AJ388770	no match	45,9	0,9	1,1
Mt92	AJ388731	no match	61,3	0,9	0,4
Mt212	AJ388846	no match	74,0	0,7	0,5
Mt193	AJ388827	no match	37,9	0,7	0,7
Mt286	AJ388916	no match	28,9	1,0	1,4
Mt169	AJ388805	no match	24,2	1,1	1,6
Mt1051	BQ079335	no match	4,9	0,6	0,8
Mt68	AJ388709	no match	277,8	0,7	0,2
Mt59	AJ388700	no match	85,8	0,8	0,2
Mt347	AJ388974	no match	14,8	0,8	0,3
Mt130	AJ388767	Leghemoglobin 1	24,9	0,2	4,6
Mt107	AJ389050	Leghemoglobin 2	172,2	0,6	0,6
Mt228	AJ388860	Leghemoglobin 3	153,5	0,8	0,9
Mt108	AJ388746	Leghemoglobin 4	375,5	0,6	0,3
Mt224	AJ388857	Leghemoglobin 6	121,3	0,8	0,8
Mt288	AJ388918	Enod 2	102,8	0,7	0,6
Mt1102	BQ079341	Enod 20	3,0	0,6	0,6
Mt309	AJ388939	Enod 40	5,6	1,0	0,9
Mt229	AJ388861	early nodulin N-75	131,9	0,5	0,6
Mt1235	BQ079362	MtN5	4,4	0,4	0,7
Mt24	AJ388672	MtN22 nodulin	140,7	0,9	1,4
Mt304	AJ388934	nms22 nodulin	100,2	0,9	0,8
Mt154	AJ388791	nodulin 25 precursor	531,4	0,4	0,2
Mt94	AJ388733	nodulin 25	82,2	0,7	0,4
Mt418	AJ389041	nodulin 26	43,9	5,4	4,0
Mt279	AJ388910	MtN27 nodulin	48,0	0,7	0,4
Mt1249	BQ079365	MtN28	6,5	1,1	1,7

Fig. 3. Comparison of gene expression profiles in galls and in nodules using macroarray analysis. **A,** Forty-four genes showing upregulation (red boxes) or downregulation (green boxes) in nodules, in galls, or in both compared to untreated root are listed. Ratios for normalized hybridization values of expression between galls 7 and 14 dpi versus roots (galls/root) and nodules 20 dpi versus roots (nodule/root) are shown. **B** and **C**, Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *CYCD3* and *NOD26* mRNA levels in galls 14 dpi induced by the nematode *Meloidogyne incognita* and in control roots are shown. Related curves for the *MTC27* constitutive control transcript are depicted below.

robot (Beckman, Fullerton, CA, U.S.A.). PCR-amplified products were used for spotting. These were made using the vector-specific 5′-oligo ATT-CGA-TGA-TGA-AGA-TAC-CCC and 3′-oligo GTA-ATA-CGA-CTC-ACT-ATA-GGG-C. The PCR products were mixed with NaOH to give a final concentration of 0.2 M NaOH. These denatured PCR products (at a concentration of 100 ng of DNA per µl) were directly used for spotting on Hybond N+ nylon membranes (Amersham, Tokyo). Each spot, containing about 15 ng of DNA, was represented three times on each membrane. Membranes were subjected to a quality control by hybridization with a nested ^{33}P-labeled oligo (GAG-TCG-ACC-CGG-G). This allowed verifying the presence of each spot, the quality of the spots, and the quality comparison amongst membranes. Macroarrays were hybridized with ^{33}P-labeled cDNA prepared from control roots, galls (7 and 14 dpi), and nodules (3 weeks old) total RNA. First strand cDNA was made as described (Györgyey et al. 2000) with the following modifications: 50 µg of total RNA was used and the incorporated label was $\alpha[^{33}P]$-dCTP. Hybridizations were done overnight at 65°C in the buffer described by Church and Gilbert (1984), and washing was done twice at 65°C with 40 mM sodium phosphate, pH 7.2, and 0.1% sodium dodecyl sulfate. The hybridization signals were analyzed with the STORM Phosphorimager, and quantification of spots was done with ArrayVision (Imaging Research Inc., St. Catharines, Canada). Results were treated in Excel (Microsoft, Redmond, WA, U.S.A.). The data obtained from different hybridizations were normalized by dividing the intensity of each dot by the average of the intensities of *MTC27*, 60S, and 40S ribosomal gene dots present on the filter to obtain a centered and normalized value. Mean and standard deviation of each spot represented three times on each membrane was calculated, and values with a variation greater than 25% were excluded from further analysis. Means were used to calculate ratios for normalized hybridization values of expression between galls and nodules compared to control roots. To determine the reproducibility of the macroarray analysis, experiments were repeated twice.

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