***Arabidopsis thaliana* Genes Expressed in the Early Compatible Interaction with Root-Knot Nematodes**

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In the compatible interaction between *Arabidopsis thaliana* and the endoparasitic nematode *Meloidogyne incognita*, galls are formed on the roots of the host plant. Differential display was used to identify alterations of gene expression in young *A. thaliana* root galls caused by *M. incognita*. Six genes were confirmed as plant genes by DNA gel blot hybridizations. Significant homology was found with a trypsin inhibitor, peroxidase, mitochondrial uncoupling protein, endomembrane protein, 20S proteasome α -subunit, and diaminopimelate decarboxylase. The cellular and temporal expression of each of the six genes was analyzed by mRNA in situ hybridizations.

Sedentary plant-parasitic nematodes have developed a highly specialized feeding relationship with their hosts and include the economically most important root-knot and cyst nematodes, *Meloidogyne*, *Heterodera*, and *Globodera* species. The infection process of these nematodes consists of a series of distinct steps (Lewis 1987). When hatched from the eggs, the vermiform infective juveniles are attracted to the host root. Root-knot nematodes usually enter the root system near the elongation zone and migrate intercellularly toward the root tip of the host plant (Wyss et al. 1992). To reach their ultimate destination, root-knot nematodes turn at the apex of the root and proceed in the direction of the vascular cylinder (Wyss et al. 1992). A small number of vascular tissue cells are selected and transformed into specialized feeding cells, called “giant cells.” These giant cells are huge and contain dense cytoplasm with large quantities of organelles, multiple lobate polyploid nuclei, a fragmented central vacuole, thickened cell walls, elaborate membrane ingrowths, and feeding tubes (Jones and Payne 1978). These features make them distinct cytologically

from the neighboring parenchymatous root cells. Giant cells achieve their multinucleated state by undergoing repeated nuclear divisions without cytokinesis (Jones and Payne 1978). The cells surrounding the giant cells hypertrophy and divide, forming a gall-like structure on the plant root.

Differential screening of cDNA libraries from nematode-infected root tissue has led to the isolation of many up-regulated plant genes. Expression of a wound-responsive gene (*pT53*), a plasmalemma proton ATPase, a serine–threonine receptor kinase, the ubiquitin-carrier protein gene (*E2*), and a *myb* transcription regulator were induced in *Meloidogyne* spp.-infected tomato roots (Bird 1996; Bird and Wilson 1994). A *Lea14*-like protein produced in tomato giant cells may protect the cytoplasmic proteins from high osmotic pressures in the feeding cells (Van der Eycken et al. 1996). Extensin, the major structural cell wall protein, probably plays a role in strengthening walls of infected and surrounding cells incorporated in the hypertrophied root gall (Niebel et al. 1993; Van der Eycken et al. 1996). *LeMir* encoding a tomato miraculin is highly induced by the root-knot nematode *Meloidogyne javanica* (Brenner et al. 1998).

The promoter of the *hmgr* gene involved with sterol biosynthesis (Cramer 1992; Cramer et al. 1993) is highly activated in developing giant cells (Bleve-Zacheo and Melillo 1997). Additionally, the promoter of the *TobRB7* gene that encodes a membrane protein believed to function as a water channel is highly induced in giant cells (Opperman et al. 1994). The *Arabidopsis thaliana* cell-cycle genes *cdc2a*, *cdc2b*, *CycA2;1*, and *CycB1;1* are induced during the early development of giant cells (de Almeida Engler et al. 1999; Niebel et al. 1996). The *atao1* gene encoding an extracellular copper amine oxidase is thought to be important in the early stages of vascular tissue development, and the *ATAO1* enzyme might have a function in cross-linking extensins or other plant cell wall proteins (Møller et al. 1998). Favery et al. (1998) found that the promoter of the *rpe* gene encoding a key enzyme in the reductive Calvin cycle and the oxidative pentose phosphate pathway is highly induced in giant cells.

In 1995, Bertoli et al. asserted that the differential-display method shows a strong bias toward highly abundant mRNAs and thus is not a suitable tool to investigate a subtle interaction such as nematode infection of plants. More recently,

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however, the first results with the use of differential display to investigate the interaction between soybean and the soybean cyst nematode were published (Hermsmeier et al. 1998). We applied differential display to identify *A. thaliana* genes that show altered mRNA levels shortly after *Meloidogyne incognita* infection. RNA subsets of young *A. thaliana* root galls isolated at five time points were displayed with those of the corresponding parts of noninfected *A. thaliana* roots to identify and isolate cDNA clones that are potentially induced by nematode infection. The characterization of six cDNA clones,

including the cellular localization of the transcripts, is described.

RESULTS

Fingerprinting the *A. thaliana*–*M. incognita* interaction.

Differential-display patterns were generated from RNA of young root galls of *A. thaliana* and the corresponding regions of noninfected roots with 19 random decamers in combination with the four possible anchor primers. In total, 27 independent differential-display bands (DiDi fragments), detectable only in lanes displaying cDNAs from root galls, were isolated and reamplified. Differential display of gall RNA at five different time points along with the corresponding controls showed that the patterns were reproducible (Fig. 1). An independent experiment with RNA isolated from a different time-course experiment in which a number of primer combinations were retested confirmed the reproducibility of the technique.

Considering that different cDNAs of the same length comigrate in a polyacrylamide gel, four to 14 individual clones of each DiDi fragment were sequenced. Indeed, several reamplified cDNA samples consisted of a mixture of different sequences. In 35% of the cases, the sequences of the different clones were identical. Seventy-four of the different sequences obtained were submitted to the EMBL database under accession nos. AJ249958–AJ249960, AJ286344–AJ286356, and AJ276712–AJ276772.

On the basis of homology search results, the DiDi clones were classified into two major categories in which several clones were studied in more detail (Table 1). Group I represents genes that could be related to plant–pathogen interactions (DiDi 4T-1, DiDi 6G-3b). Group II consists of genes involved in cell growth and metabolism (DiDi 8A-1a, DiDi 8C-5d, DiDi 17A-2b, and DiDi 18A-1f).

Further characterization of the differential-display clones included DNA gel blot hybridizations, RNA gel blot hybridizations, reverse transcription-polymerase chain reaction (RT-PCR) experiments, and mRNA in situ localization. To investigate the origin of the DiDi clones, genomic DNA gel blot hybridizations were performed (Fig. 2). The large majority of the isolated cDNAs correspond to plant genes (80%). Fourteen percent of the cDNA clones hybridized to plant and nematode DNA and 5% were of nematode origin.

RNA gel blot experiments were performed to confirm the differential expression of the DiDi clones in nematode-infected *A. thaliana* roots. Of the 17 cDNA clones tested, two (DiDi 6G-3b and DiDi 8C-5d) gave a signal that was strong enough to be detected on a RNA gel blot. DiDi 6G-3b transcripts were clearly up regulated in nematode-infected roots compared with noninfected control roots (Fig. 3). The signal

Fig. 1. Differential display gel showing the amplification products of RNA from *Arabidopsis thaliana* root galls harvested 2 (2i), 3 (3i), 4 (4i), 5 (5i), and 7 (7i) days postinoculation with *Meloidogyne incognita* and from noninfected control roots (c lanes). Amplification reactions were done with decamer 6 (5'-GCAATCGATG-3') and anchor primer T₁₂MG. Arrowhead indicates the differentially displayed product designated DiDi 6G-3b. Indicated sizes are estimates.

Table 1. Characteristics of selected cDNA fragment^a

Clone	Accession no.	Primers	DiDi fragment length (bp)	Homology ^a	P ^b
DiDi 4T-1	AJ249958	T ₁₂ MT-AP-4	205	Trypsin inhibitor (I = 100%) AC002335	8.6e-50
DiDi 6G-3b	AJ286345	T ₁₂ MG-AP-6	330	Peroxidase ATP6a (I = 98%) Z99708	8.8e-228
DiDi 8A-1a	AJ286346	T ₁₂ MA-AP-8	350	Mitochondrial uncoupling protein (I = 100%) AL035356	2.8e-24
DiDi 8C-5d	AJ249959	T ₁₂ MC-AP-8	400	Endomembrane protein (I = 55%) AC006932	4.8e-15
DiDi 17A-2a	AJ286351	T ₁₂ MA-AP-17	270	20S proteasome α 7-subunit (I = 100%) AF043528	e-108
DiDi 18A-1f	AJ249960	T ₁₂ MA-AP-18	635	Diaminopimelate decarboxylase (I = 43%) AE000728	2.2e-23

^a I = identity at the amino acid level.

^b P = smallest sum probability.

of the DiDi 8C-5d mRNAs became visible only after long exposure times and was slightly higher in nematode-infected roots than in control roots (data not shown).

As a substitute for RNA gel blot analyses, semiquantitative RT-PCR was used to compare mRNA samples from control and infected roots (data not shown). The expression of 15 clones was induced significantly in nematode-infected roots, whereas the other clones were induced weakly, expressed constitutively, or not tested. Six plant DiDi clones (Table 1) were studied at different time points via mRNA in situ hybridization experiments in nematode-infected and noninfected control roots of *A. thaliana*. The cellular expression patterns are summarized in Table 2 and discussed below.

Cellular and temporal mRNA patterns of DiDi 4T-1 encoding a serine proteinase inhibitor.

In mRNA in situ localization experiments, sections of *A. thaliana* root galls of 3, 7, 14, and 21 days were hybridized to ³⁵S-labeled sense and antisense probes of DiDi 4T-1. At the earliest time point tested (3 days postinoculation [dpi]), a very high DiDi 4T-1 mRNA concentration was seen in the endodermal cells surrounding the giant cells (Fig. 4A) compared with that in noninfected *A. thaliana* roots (Fig. 4F). Approximately the same expression pattern was detected in 1-week-old root galls (Fig. 4B), and sometimes a weak signal was visible in young giant cells. In contrast, at 14 dpi the signal in the giant cells was clear but below detection in the endodermis (Fig. 4C). In 21-day-old galls, the mRNA level of DiDi 4T-1 in the giant cells had decreased and no signal was ob-

served in the endodermis (Fig. 4D) nor in the other gall tissues, except for those of lateral roots emerging from the gall (Fig. 4E). In noninfected control roots, no signal could be detected (Fig. 4F). Hybridizations of the root sections with a sense transcript of DiDi 4T-1 did not reveal a signal at the different time points tested (Fig. 4G).

Cellular and temporal mRNA patterns of DiDi 6G-3b encoding a peroxidase.

Upon RNA gel blot hybridization with the DiDi 6G-3b clone, a transcript of approximately 1.2 kb clearly was up regulated at 3 and 14 dpi with *M. incognita* compared with the control roots (Fig. 3). mRNA in situ hybridizations revealed a high level of DiDi 6G-3b mRNA in young giant cells (Fig. 4H) and in the endodermal cells of 3-day-old root galls (data not shown). In 1-week-old giant cells, the mRNA level of DiDi 6G-3b was still high, similar to that in 3-day-old feeding cells (Fig. 4I). Additionally, the endodermal cells surrounding the feeding cells highly expressed the peroxidase at this time point (Fig. 4I). Two- and 3-week-old giant cells did not show any signal. The DiDi 6G-3b gene was still active in the endodermal cells of 2- and 3-week-old root galls (Fig. 4J), although transcripts were found in fewer root sections than in 3-day-old feeding sites. DiDi 6G-3b also was expressed at the base of young side roots emerging from nematode infection sites (Fig. 4K). In noninfected control roots, no clear hybridization signal of DiDi 6G-3b could be detected (Fig. 4L). Furthermore, no signal was observed in nematode feeding sites or control roots after hybridization with a sense transcript of DiDi 6G-3b (data not shown).

Fig. 2. DNA blot hybridization of *Arabidopsis thaliana* (P) and *Meloidogyne incognita* (N) with six selected DiDi clones. In all cases, 5 µg of genomic DNA digested with *Hind*III was separated on 1% agarose gel, transferred to a Hybond-N nylon membrane, and hybridized to ³²P-labeled DiDi clones. Phage λ DNA digested with *Pst*I was used as a molecular weight marker. A, DiDi 4T-1; B, DiDi 6G-3b; C, DiDi 8A-1a; D, DiDi 8C-5d; E, DiDi 17A-2b; and F, DiDi 18A-1f.

Cellular and temporal mRNA patterns of DiDi 8A-1a encoding a mitochondrial uncoupling protein.

In situ hybridizations revealed a high level of DiDi 8A-1a mRNA in the endodermal cells bordering young root galls (3 and 7 dpi) (Fig. 5A and B). In older root galls (14 dpi), the DiDi 8A-1a transcript was found inside the giant and gall parenchyma cells (Fig. 5C). The mRNA level of DiDi 8A-1a was high (Fig. 5D), especially in fully developed giant cells (21 dpi). In endodermal cells of 2- and 3-week-old galls, a DiDi 8A-1a mRNA signal could no longer be detected. The base of young lateral roots emerging from infection sites showed DiDi 8A-1a mRNA hybridization in the endodermal cells (Fig. 5E). A faint signal could be localized in the root tips of some noninfected control roots (Fig. 5F), confirming the basal level of DiDi 8A-1a expression seen on the RT-PCR patterns (data not shown). Hybridization with a sense transcript of DiDi 8A-1a never revealed a clear signal (Fig. 5G).

Cellular and temporal mRNA patterns of DiDi 8C-5d encoding a putative endomembrane protein.

The expression of DiDi 8C-5d was followed during the postinfection period (2, 6, 10, 15, and 21 dpi with *M. incognita*) via RNA gel blot analysis.

After a 1-week exposure period, a 1-kb DiDi 8C-5d transcript was observed in noninfected control roots, whereas a higher level was seen in infected roots from 6 dpi onward, with a maximum at 10 dpi (data not shown).

mRNA in situ hybridizations demonstrated that the hybridization signal of DiDi 8C-5d was localized specifically in the giant cells of young root galls (3 dpi) (Fig. 5H). In 7-day-old root galls, DiDi 8C-5d transcripts could be seen in the giant cells and even more strongly in the endodermal cells (Fig. 5I). Later, in the development of the root gall, the signal was located higher inside the nearly completely expanded giant cells (Fig. 5J). Some root sections of 14-day-old root galls still showed a low DiDi 8C-5d hybridization signal in the endodermal cells (data not shown). Three weeks after inoculation, DiDi 8C-5d mRNA was found inside the giant cells but not in the endodermis (data not shown). In noninfected and infected *A. thaliana* roots, a distinct signal was visible in the root tips (Fig. 5K). Root tissues hybridized to the control sense transcript of DiDi 8C-5d did not reveal any signal (data not shown).

Table 2. In situ hybridization of the selected cDNA fragments in nematode-induced giant cells, other gall tissues, and noninfected control roots

Clone	Expression in giant cells ^a				Expression in other gall tissues ^b	Control
	3 dpi	7dpi	14 dpi	21 dpi		
DiDi 4T-1	+	+	++	+	Endodermis (3 and 7 dpi); lateral roots at NFS	-
DiDi 6G-3b	+++	+++	-	-	Endodermis (3, 7, 14, and 21 dpi); base of lateral roots at NFS	-
DiDi 8A-1a	-	-	+++	+++	Endodermis (3 and 7 dpi); parenchyma; base of lateral roots at NFS	Some root tips
DiDi 8C-5d	++	++	+++	++	Endodermis (7 and 14 dpi); root cap	Root cap
DiDi 17A-2a	+	++	+++	+++	Endodermis (7 and 14 dpi); young root tips	Young root tips
DiDi 18A-1f	+++	+++	+	+	Pericycle (3 and 7 dpi)	Pericycle

^a - = no expression; + = weak expression; ++ = strong expression; +++ = very strong expression; dpi = days postinoculation.

^b NFS = nematode feeding site.

Fig. 3. RNA blot hybridization from *Arabidopsis thaliana* root galls at 3 (3i) and 14 (14i) days postinoculation and from *A. thaliana* noninfected control roots (3c and 14c) with a DiDi 6G-3b probe. **A**, Arrowhead indicates the 1.35-kb transcript. **B**, Gels stained with ethidium bromide to show equal loading of RNA.

Cellular and temporal mRNA patterns of DiDi 17A-2a encoding a 20S proteasome α -subunit.

In situ hybridizations demonstrated a low level of DiDi 17A-2a mRNA in giant cells of 3-day-old galls (data not shown). One week upon inoculation, a significant accumulation of DiDi 17A-2a transcripts was observed in the giant (Fig. 6A) and endodermal cells (Fig. 6B). Later on, in gall development, the transcript level of DiDi 17A-2a was higher inside the giant cells (Fig. 6C). DiDi 17A-2a mRNA was present in the endodermal cells of 2-week-old galls, but in fewer sections than observed in 1-week-old galls. The DiDi 17A-2a transcript was very abundant in giant cells but could no longer

be detected in endodermal cells of 21-day-old *A. thaliana* root galls (Fig. 6D, overexposed). Young noninfected root tips and very young lateral roots emerging from the infection sites showed a faint hybridization signal of DiDi 17A-2a (Fig 6E). Hybridization with a sense transcript of DiDi 17A-2a, however, never revealed a signal (Fig. 6F).

Cellular and temporal mRNA patterns of DiDi 18A-1f homologous to a diaminopimelate decarboxylase.

In situ hybridizations showed a high level of DiDi 18A-1f transcript inside 3-day-old giant cells and in the pericycle cells surrounding the giant cells (Fig. 6G). Similar expression

Fig. 4. In situ localization of DiDi 4T-1 and DiDi 6G-3b transcripts in *Arabidopsis thaliana* roots infected with *Meloidogyne incognita* and noninfected control roots. Longitudinal and cross sections were hybridized to 35 S-labeled DiDi 4T-1 sense (G) and antisense RNA probes (A to F) or to a DiDi 6G-3b antisense RNA probe (H–L). Root tissue was stained with a 0.05% toluidine blue solution. **A and E**, Bright-field micrographs (signal seen as black dots); **B–D and F–L**, Dark-field micrographs (signal seen as white dots). DiDi 4T-1 transcripts are visible in **A**, endodermal cells of 3-day-old root galls; **B**, endodermal cells of 7-day-old root galls; **C**, 14-day-old giant cells; **D**, 21-day-old giant cells; and **E**, endodermal cells of lateral roots emerging from the gall. **F**, Control root hybridized to antisense probe and **G**, gall tissue (3 days postinoculation [dpi]) hybridized to the sense probe. DiDi 6G-3b transcripts are visible in **H**, 3-day-old giant cells; **I**, 7-day-old giant cells and endodermal cells of 7-day-old root galls; **J**, endodermal cells of 21-day-old root galls; and **K**, at the base of lateral roots emerging from young infection sites (3 dpi). **L**, Control root tissue. e = endodermis; gc = giant cell; n = nematode. Bar = 50 μ M.

Fig. 5. In situ localization of DiDi 8A-1a and DiDi 8C-5d transcripts in *Arabidopsis thaliana* roots infected with *Meloidogyne incognita* and in noninfected control roots. Longitudinal and cross sections were hybridized to ^{35}S -labeled DiDi 8A-1a sense (G) and antisense RNA probes (A–F) or to a DiDi 8C-5d antisense RNA probe (H–K). Root tissue was stained with a 0.05% toluidine blue solution. **A and F**, Bright-field micrographs (signal appears as black dots); **B–E and G–K**, Dark-field micrographs (signal appears as white dots). DiDi 8A-1a transcripts are visible in endodermal cells of **A**, 3-day-old and **B**, 7-day-old root galls; **C**, 14-day-old giant cells and parenchyma cells of 14-day-old root galls; **D**, 21-day-old giant cells; **E**, endodermal cells of young lateral roots emerging from infection sites; and **F**, some root tips of noninfected control roots. **G**, Gall tissue (14 days postinoculation) hybridized to the sense probe. **H**, DiDi 8C-5d transcripts are visible in 3-day-old giant cells; **I**, giant cells and endodermal cells of 7-day-old root galls; **J**, 14-day-old giant cells and **K**, the root cap of (lateral, in this case) control roots. e = endodermis; gc = giant cell; n = nematode. Bar = 50 μM .

patterns were visible in 1-week-old root galls (data not shown). The level of DiDi 18A-1f transcript in the giant cells of 2-week-old root galls was lower than observed in young giant cells (Fig. 6H) and, in 3-week-old giant cells, DiDi 18A-1f transcripts were only detectable after longer exposure times (Fig. 6I). DiDi 18A-1f mRNA was no longer detectable in the pericycle cells of 2- and 3-week-old galls (Fig. 6H and I). A weak but clear signal of DiDi 18A-1f was visible in the pericycle cells of noninfected control roots of *A. thaliana* (Fig. 6J). Hybridizations of root sections with the sense probe of DiDi 18A-1f gave no signal (data not shown).

DISCUSSION

Up-regulated genes in nematode-infected *A. thaliana* roots.

The differential-display technique was chosen to isolate genes involved in the early stages of plant–nematode interactions (Liang and Pardee 1992). The generated differential-display patterns were highly reproducible. Twenty-seven independent cDNAs that appeared only in lanes containing cDNA from infected material were isolated, and 74 different sequences were cloned. DNA gel blot hybridizations on 55 clones demonstrated that the majority of the isolated clones represent plant genes (44 clones). Remarkably, only three clones are of nematode origin, which is probably a result of the method used for RNA extraction. Some cDNA fragments hybridized to plant and nematode DNA (eight clones), possibly because DiDi fragments are often short, AT-rich 3' cDNA ends that hybridize to both DNA sources.

We found a clear homology in the database for 25 of the isolated plant clones, which were then grouped into two categories. A general defense mechanism, revealed by the enhanced expression of proteinase inhibitors (DiDi 4T-1) and peroxidases (DiDi 6G-3b), has been described for incompatible and compatible plant–fungus (Curtis et al. 1995; Reimers et al. 1992; Ryan 1990; Terras et al. 1993) and plant–bacteria interactions (Koiwa et al. 1997; Lummerzheim et al. 1993).

Obvious characteristics of nematode-induced feeding cells are a highly active metabolism and enormous cell expansion. Genes encoding housekeeping proteins such as those involved in translation, different metabolic pathways, mitochondrial functioning, or protein transport and turnover have been found via differential display to be up regulated upon nematode infection, i.e., a mitochondrial uncoupling protein (DiDi 8A-1a), endomembrane protein (DiDi 8C-5d), proteasome (DiDi 17A2a), and diaminopimelate decarboxylase (DiDi 18A-1f).

Expression of DiDi 4T-1 and DiDi 6G-3b considered part of a wound response toward plant-pathogenic nematodes.

Proteinase inhibitors (Ryan 1990) are thought to play a major role as a natural phytochemical defense mechanism against a variety of microbial pathogens and herbivory predators (Green and Ryan 1972). Proteinase inhibitor gene expression is induced by wounding (Pautot et al. 1991) and is developmentally regulated in flowers of tomato and potato plants (Peña-Cortés et al. 1991). DiDi 4T-1 encoding a trypsin inhibitor was expressed mainly in the endodermal cells surrounding the expanding giant cells.

The development of the giant cells and the growing nematodes cause a high stress condition inside the plant root, disordering the cells of the vascular cylinder and the pericycle. The

endodermis surrounding the central stele is a tightly arranged cell layer that forms a protective barrier and keeps the root cells together. The expression of a trypsin inhibitor in nematode-affected endodermal cells can be seen as a part of a wound reaction of the host plant.

Peroxidases clearly play a role in plant–pathogen interactions (Lummerzheim et al. 1993; Mohan et al. 1993; Rebmann et al. 1991; Ye et al. 1990). Peroxidases have a function in secondary cell wall biosynthesis by polymerizing phenolic residues into wall polymers (Mohan et al. 1993) or by forming rapid cross links among cellulose, hydroxy–proline-rich glycoproteins, and lignin (Bradley et al. 1992; Lagrimini and Rothstein 1987; Roberts et al. 1988; Walter 1992; Ye et al. 1990). In situ hybridizations of DiDi 6G-3b in nematode-infected root tissue show an induction of the peroxidase gene inside giant cells and, even more pronounced, in the endodermal cells.

Peroxidases might play a significant role in the strengthening of the plant cell wall and may help to fortify the plant root upon nematode infection. The presence of the expanding juveniles causes a swelling of the plant root and, consequently, an enormous pressure inside the host-root system. Böckenhoff and Grundle (1994) reported that the turgor inside the feeding sites of *Heterodera schachtii* is 2.5-fold higher than in normal vascular parenchymatous cells.

High levels of DiDi 8A-1a correlated with the presence of numerous mitochondria in giant cells.

A plant-uncoupling mitochondrial protein (PUMP) of potatoes was induced by cold treatment and may be relevant for thermoregulation in plants (Laloi et al. 1997). During some of the processes triggered by a burst of respiration when a sudden cutoff of ATP production is observed, e.g., rapid growth, flowering, seed formation, and fruit ripening, the temperature of the plant can be elevated above the surrounding temperature (Vercesi et al. 1995).

In the early days of research in plant–nematode interactions, many articles were published on the ultrastructure of giant cells. A striking increase of cell organelles, including mitochondria, inside the metabolically active giant cells also was reported (Huang 1985; Jones and Payne 1978). PUMP-expression enhancement upon nematode infection (DiDi 8A-1a) implies an increase in the number of mitochondria in the affected cells or an increase in the number of uncoupling proteins per mitochondrion. It also could be a result of the combination of the two possible events.

High DiDi 8C-5d mRNA levels in root tips and giant cells.

The peptide encoded by DiDi 8C-5d was homologous to the human endomembrane protein (hMP70). These proteins are thought to play an essential role in protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Schimmöller et al. 1995). In plants, the secretory pathway also is responsible for the transport of many cell wall precursor molecules (Vian and Roland 1991), membrane-bound enzymes (Haigler and Brown 1986), and various ion pumps (Hager et al. 1991; Satiat-Jeunemaitre and Hawes 1993). Another important function of the secretory pathway is to increase the surface area of the plasma membrane when the cell is growing.

In developing nematode feeding cells, extensive cell wall thickening and cell wall ingrowths have been observed. Addi-

tionally, the surface of the plasma membrane lining the cell wall must increase. The fusion of ER-derived and Golgi-processed vesicles to the plasma membrane can deliver the necessary components to the expanding nematode feeding cells.

DiDi 8C-5d transcripts also were detected in root cap cells of noninfected and infected root tips. Not all polysaccharides made and secreted by the Golgi apparatus are destined for the cell wall. A fucose-rich mucilage is produced in the Golgi complexes of root cap cells and transported across the plasma membrane (Bar-Peled et al. 1995; Campbell and Greaves 1990).

DiDi 17A-2b possible involved in protein homeostasis of galls.

The ability of cells to switch from one developmental state to another or to adapt to new environmental conditions requires a rapid turnover in existing cellular activities, a process which often is dependent on proteolysis. A highly selective and regulated mechanism of protein breakdown is handled by the proteasome (Vierstra 1996). In normal *A. thaliana* roots, the 20S proteasome is expressed at a very low level (Fu et al. 1998). A high rate of protein synthesis, with or without a specific and/or temporal function in the development of giant cells, implies a high turnover of these proteins. The cellular expression pattern of DiDi 17A-2b inside the giant cells and endodermis of infected roots fits with the observation that many genes are up regulated in these cell types.

DiDi 18A-1f shares homology with diaminopimelate decarboxylase.

The biosynthesis of lysine generally follows one of two possible pathways. The aminoadipic acid pathway is restricted to fungi and euglenoid flagellates. Bacteria, higher plants, blue-green and green algae, and some fungi utilize the diaminopimelic acid pathway (Mazelis et al. 1976; Mazelis et al. 1977). The last step in this pathway comprises the decarboxylation of *meso*-2,6-diaminopimelate. Lysine metabolism in *A. thaliana* is regulated highly by complex biochemical, physiological, and environmental controls (Ben-Tzvi Tzchori et al. 1996).

An enhancement of diaminopimelate decarboxylase expression could implicate an increased lysine content in giant cells. Taking into account that giant cells are very active metabolically, an increase in amino acid level, similar to that of lysine, could be true for any other amino acid.

Different expression patterns throughout gall development.

In a number of articles, different genes isolated by the screening of cDNA libraries (Bird 1996; Bird and Wilson 1994; Brenner et al. 1998; Niebel et al. 1993; Van der Eycken et al. 1996) or by differential display (Hermsmeier et al. 1998) were assumed to be up regulated in nematode-induced galls. Here, identified clones are up regulated in nematode-induced galls and their cellular expression patterns are described. In situ hybridizations revealed that, in addition to high mRNA levels of the DiDi clone under investigation in nematode feeding cells, the genes also were highly up regulated in other root gall tissues such as the endodermis or the pericycle. The cellular expression patterns of the various DiDi clones also

differed in time throughout the development of the root gall and, consequently, the expression of the DiDi clones represent more than a higher level of total RNA in nematode-infected cells (Table 2). Considering the in situ hybridization patterns in relation to nematode infections of plant roots, one can speculate on the possible functions of the DiDi clones in giant cell formation and root gall development, although further analyses should be performed to verify the role of the isolated genes.

We conclude that the isolation of a substantial number of nematode-induced plant genes clearly demonstrates that the differential-display technique is a very powerful method to dissect the *A. thaliana*-*M. incognita* interaction at the molecular level, although the cellular expression pattern of the clones should be determined by mRNA in situ hybridizations or other tests.

MATERIALS AND METHODS

Biological material.

Roots of tomato (*Lycopersicon esculentum* L.) cv. Marmande transformed with *Agrobacterium rhizogenes* and grown on Gamborg's B5 medium (Duchefa, Haarlem, The Netherlands) at 28°C were used to rear *M. incognita* race I nematodes. Stage 2 infective juveniles were obtained from egg masses hatched in sterile water 6 to 8 weeks after inoculation.

Eighteen-day-old *A. thaliana* (L.) Heynh. ecotype Columbia seedlings grown in Falcon 1005 petri dishes (Becton-Dickinson, Bedford, MA, U.S.A.), filled with Knop medium (Knop 1860), were inoculated with approximately 50 *M. incognita* stage 2 juveniles per plant. Root galls were dissected from 500 plant roots per time point with a Wild stereoscopic microscope (Heerbrugg, Germany).

RNA extraction.

Total RNA from plant tissues was extracted via a mini-RNA preparation method based on LiCl precipitation. Galls dissected from the roots were frozen quickly under liquid nitrogen simultaneously with the corresponding noninfected root sectors from control plants. Of the collected root tissue, 50 to 100 mg was ground in a mortar under liquid nitrogen. The powder was transferred to a 1.5-ml Eppendorf micro test tube (Hamburg, Germany) and extracted with 0.5 ml of extraction buffer (0.2 M Tris, pH 7.5; 0.1 M LiCl; 5 mM EDTA; 1/10 vol of 10% sodium dodecyl sulfate [SDS]). After two phenol-chloroform (24:1) extractions, the aqueous phase was precipitated with 1 vol of 6 M LiCl. The pellet was washed with 3 M LiCl and resuspended in diethylpyrocarbonate-treated water. A final overnight precipitation at -80°C was performed with 0.1 vol of 3 M NaOAc (pH 4.8) and 2 vol of 100% ethyl alcohol. Poly(A)+ RNA was prepared with the Dynabeads biomagnetic separation system (Dynal, Oslo, Norway).

Differential-display procedure.

All components, except *Taq* DNA polymerase (Perkin Elmer, Norwalk, CT, U.S.A.) and radiolabeled dATP (Amersham Pharmacia Biotech, Little Chalfont, U.K.), were acquired from Genhunter (Brookline, MA, U.S.A.) in commercially available RNAmapping kits. Total RNA prepared from *A. thaliana* root galls harvested 2, 3, 4, 5, and 7 dpi and obtained from the corresponding noninfected *A. thaliana* roots

Fig. 6. In situ localization of DiDi 17A-2b and DiDi 18A-1f transcripts in *Arabidopsis thaliana* roots infected with *Meloidogyne incognita* and noninfected control roots. Longitudinal and cross sections were hybridized to ^{35}S -labeled DiDi 17A-2b sense (F) and antisense RNA probes (A–E) and a DiDi 18A-1f antisense RNA probe (G–J). Root tissue was stained with a 0.05% toluidine blue solution. A–J, Dark-field micrographs (signal appears as white dots). DiDi 17A-2b transcripts are visible in A, 7-day-old giant cells; B, endodermal cells of 7-day-old root galls; C, 14-day-old giant cells; D, 21-day-old giant cells (overexposed); and E, the root tip (arrow), also of control plants. F, Control root tissue hybridized to the sense probe. DiDi 18A-1f transcripts are visible in G, 3-day-old giant cells and pericycle cells of 3-day-old root galls; H, 14-day-old giant cells; I, 21-day-old giant cells (overexposed); and J, pericycle cells of noninfected control roots. gc = giant cell; n = nematode; p = pericycle. Bar = 50 μm .

was DNase I treated as described by Sambrook et al. (1989) with RQ1 DNase (Promega, Madison, WI, U.S.A.). Differential-display patterns obtained from poly(A)⁺ RNA and total RNA were essentially identical. Total RNA (500 ng) was reverse transcribed in four subsets with four different oligo(dT) primers (T₁₂MA, T₁₂MC, T₁₂MG, and T₁₂MT; M indicates dA, dC, or dG) anchored to the 5' end of the poly(A) tail of the mRNA (Liang et al. 1993) in a 20- μ l reaction volume. Subsequently, 1 μ l of first-strand cDNA was amplified via PCR with the appropriate anchor primer and an arbitrary decamer in the presence of radiolabeled dATP in a 50- μ l PCR mixture according to the manufacturer's instructions. Annealing of the primers was most efficient at 40°C. Of the PCR mixtures, 5.5 μ l were separated on 4.5 and 6% denaturing polyacrylamide gels. After exposure (24 or 48 h at -70°C), differentially expressed cDNAs were recovered from the dried gel and amplified according to the manufacturer's guidelines. The cDNAs were blunt ended with the Klenow fragment of DNA polymerase (Roche Diagnostics, Brussels, Belgium) and phosphorylated with T4 DNA kinase (Roche Diagnostics). Subsequently, the cDNA fragments were cloned in a dephosphorylated pBluescript II KS(-) vector (Stratagene, La Jolla, CA, U.S.A.). The cloned differential-display fragments were designated DiDi fragments and specified by symbols, indicating the primer combination used to generate the fragment (before the dash) and the clone (behind the dash).

DNA sequence analysis.

DNA sequencing procedures were carried out with a modified version of the method of Sanger et al. (1977), incorporating fluorescent-labeled dideoxy nucleotides. DNA sequence data were assembled and compared with nucleic acid (Blast + Beauty) and amino acid sequences (BlastX + Beauty) in various databases (EMBL, GenBank, and Swiss-Prot).

DNA gel blot hybridization and probe preparation.

To discriminate plant from nematode cDNA clones, genomic DNA gel blot analyses were performed. *A. thaliana* DNA was prepared according to Dellaporta et al. (1983), with some modifications. Nematode DNA was isolated as described by Emmons et al. (1979).

Genomic DNA (5 μ g) from both sources was digested overnight at 37°C with *Hind*III (Amersham Pharmacia Biotech), separated on 1% agarose gel, transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech), and hybridized overnight at 65°C (3 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], pH 7.0; 0.25% milk powder; 0.1% SDS; and 0.002% denatured herring sperm DNA) with [α -³²P] dCTP-labeled probes synthesized from the differential-display clones. The probes were generated with the T7 Quickprime kit (Amersham Pharmacia Biotech), according to the manufacturer's protocol. Washing steps (15 min each) were performed at 65°C, once in 3 $\times$ SSC, 0.1% SDS; twice in 1 $\times$ SSC, 0.1% SDS; and once in 0.1 $\times$ SSC, 0.1% SDS. Blots were exposed to scientific X-OMAT imaging films at -70°C for 1 week (Kodak, Rochester, NY, U.S.A.).

RNA gel blot hybridization and probe preparation.

Total RNA (12 μ g) (prepared as described above) was separated on a denaturing 1% agarose gel (containing 2% formaldehyde), transferred to a Hybond-N nylon membrane

and hybridized overnight at 65°C (0.5 M sodium phosphate buffer, pH 7.4; 1% bovine serum albumin; 7% SDS) with probes generated from the differential-display clones. Antisense riboprobes were synthesized with T3-T7 Riboprobe in vitro transcription systems (Promega) according to the manufacturer's protocol. Hybridizations were performed with the full-length cDNA of DiDi 6G-3b and with the DiDi fragment in all other cases. Unbound probe was washed away at 65°C, twice for 15 min in 2 $\times$ SSC, 0.1% SDS; twice for 15 min in 1 $\times$ SSC, 0.1% SDS; and once for 15 min in 0.1 $\times$ SSC, 0.1% SDS. To verify the integrity and equal loading of the RNA, spectrophotometrical measurements were performed and ethidium bromide was added to the samples, allowing visualization of the RNA. The transfer of the RNA to the membrane was checked by methylene blue staining according to Sambrook et al. (1989).

mRNA in situ hybridization.

Root galls of *A. thaliana* were dissected 3, 7, 14, and 21 dpi with *M. incognita* and fixed overnight at 4°C in 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2). *A. thaliana* control roots were collected simultaneously. After dehydration, the root tissue was embedded in paraplast (Sigma-Aldrich Chemical, St. Louis, MO, U.S.A.). Thin, 10- μ m sections (microtome HM 360, Microm, Walldorf, Germany) were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma). Slides were hybridized to a ³⁵S-labeled sense and antisense transcript of the cDNA clone of interest, generated with T7, T3, or SP6 RNA polymerase Riboprobe kit. After hybridization (de Almeida Engler et al. 2001), the slides were washed at room temperature for 1 h in 2 $\times$ SSC and for 1 h at 42°C in a 50% 0.1 $\times$ SSC–50% formamide solution. Autoradiography was performed by dipping the slides in NBT-2 Kodak emulsion (Sigma) and exposing them at 4°C until a signal was detected (3 to 6 weeks). After developing the slides, they were stained with a 0.05% toluidine blue solution in tap water and mounted with Depex (Sigma). Light microscopy analysis and photography was performed with a Diaplan microscope (Leitz, Wetzlar, Germany). The hybridization signal was investigated by bright-field (signals seen as black dots) and dark-field (signals seen as white dots) microscopy.

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