Changes in mRNA Abundance within *Heterodera schachtii*-Infected Roots of *Arabidopsis thaliana*

Dieter Hermsmeier,¹ Jennifer K. Hart,¹ Marina Byzova,¹ Steven R. Rodermel,² and Thomas J. Baum¹

¹Department of Plant Pathology, and ²Department of Botany, Iowa State University, Bessey Hall, Ames 50011, U.S.A.

Accepted 5 November 1999.

Gene expression changes in plant roots infected by plant-parasitic cyst nematodes are involved in the formation of nematode feeding sites. We analyzed mRNA abundance changes within roots of *Arabidopsis thaliana* during the early compatible interaction with *Heterodera schachtii*, the sugarbeet cyst nematode. Approximately 1,600 root sections, each containing a single parasitic nematode and its feeding site, and 1,600 adjacent, nematode-free root sections were excised from aseptic *A. thaliana* cultures 3 to 4 days after inoculation with *H. schachtii*. These tissue samples were termed infected and uninfected, respectively. Preparasitic nematodes were added to the uninfected tissue sample to maintain the nematode to plant tissue proportion. mRNA extracted from these two tissue samples was subjected to differential display analysis. Thirty-six cDNA clones corresponding to mRNA species with different abundance between both tissue samples were isolated. Of these clones, 24 were of *A. thaliana* origin and 12 were from *H. schachtii*. Differential display data predicted that the *A. thaliana* cDNA clones corresponded to 13 transcripts that were more abundant in the infected root sections and 11 transcripts that were more abundant in the uninfected root sections. *H. schachtii* cDNA clones were predicted to correspond to four transcripts that were more abundant in parasitic nematodes and to eight transcripts that were more abundant in preparasitic nematodes. In situ hybridization experiments confirmed the mRNA abundance changes in *A. thaliana* roots predicted by the differential display analyses for two *A. thaliana* clones.

Additional keywords: AP2, AtEBP, EREBP, RAP2.3, syncytium.

Cyst nematodes (*Heterodera* and *Globodera* spp.) cause substantial damage to a variety of crops worldwide (Atkinson

1995; Sasser and Freckman 1987; Wrather et al. 1997). Infective second-stage juveniles (J2) of cyst nematodes hatch from eggs in the soil and invade roots of nearby host plants. After root penetration, the nematodes tunnel through the root cortex with the aid of cellulolytic enzymes (de Boer et al. 1999; Smant et al. 1998; Wang et al. 1999), breaking through cell walls and causing necrosis as they travel (Golinowski et al. 1996). A nematode then selects an initial feeding cell in the root vascular cylinder and uses its protrusible mouth spear (stylet) to inject esophageal gland secretions into or around this cell (Hussey 1989; Williamson and Hussey 1996). These secretions are thought to act as molecular signals to trigger physiological and morphological changes in the plant (Williamson and Hussey 1996). These changes include the fusion of the initial feeding cell with neighboring cells to form a feeding site, or syncytium, which is a metabolic sink serving to nourish the developing nematode (Sijmons et al. 1994). As sedentary obligate endoparasites, cyst nematodes depend entirely on functional syncytia to complete their life cycles. The formation of the syncytium is thought to be the result of nematode-induced alterations in plant gene expression (Williamson and Hussey 1996). For this reason, the identification of promoter sequences and plant genes that change expression in the vicinity of developing syncytia is of prime interest (Barthels et al. 1997; Favery et al. 1998; Goddijn et al. 1993; Govere et al. 1998; Gurr et al. 1991; Hermsmeier et al. 1998; Møller et al. 1998; Niebel et al. 1996; Puzio et al. 1998).

In this paper, we present our progress toward the identification of *Arabidopsis thaliana* gene expression changes involved in the formation of syncytia in response to infection by *Heterodera schachtii*, the sugarbeet cyst nematode. As in any research project that seeks to explore developmental processes, the experimental design is of utmost importance to avoid disturbing influences stemming from unrelated events. In our case, gene expression due to systemic wound or defense reactions of plants under nematode attack needed to be minimized. We addressed this issue by comparing root sections harboring developing syncytia with immediately adjacent root sections without syncytia. Because both samples were from infected roots, systemic wound or defense responses should have been active at similar levels in both samples.

We used the technique of differential display of mRNA (Liang and Pardee 1992) to analyze mRNA abundance as a first indicator of gene expression changes. Here we report the

Corresponding author: Thomas J. Baum; Telephone: 1-515-294-2398; Fax: 1-515-294-9420; E-mail: tbaum@iastate.edu

Current address of Dieter Hermsmeier: Max-Planck-Institut für Chemische Ökologie, Tatzendpromenade 1A, 07745 Jena, Germany.

Current address of Marina Byzova: Plant Genetic Systems N.V., Jozef Plateastraat 22, B 9000, Ghent, Belgium.

Dieter Hermsmeier and Jennifer K. Hart contributed equally to this manuscript.

identification of *A. thaliana* cDNA clones that correspond to mRNA species that change abundance specifically around the developing syncytium. We also identified *H. schachtii* cDNA clones that correspond to mRNA abundance changes occurring during the transition from the preparasitic (i.e., before root penetration) to the parasitic (i.e., after root penetration) life style. We have confirmed the predicted abundance changes of two *A. thaliana* cDNA clones via in situ hybridization.

RESULTS

Differential display analyses.

We used monoxenic cultures of *H. schachtii* on *A. thaliana* to obtain tissue samples suitable for differential display analyses. The locations of developing syncytia of *H. schachtii* were identified by sedentary nematodes protruding from the *A. thaliana* roots at 3 to 4 days after inoculation. Approximately 1,600 root sections harboring individual syncytia, each with a single parasitic J2 attached, were excised. Next, equally sized root sections were cut immediately adjacent to each syncytium section. These samples were called the infected and uninfected root sections, respectively. Preparasitic J2 were added to the uninfected root sections in a 1:1 ratio of nematodes to root sections. mRNA from both samples was subjected to differential display analysis as described in Materials and Methods. A total of 36 cDNA clones corresponding to bands with intensity differences in the differential display analyses were isolated.

High-stringency DNA-blot hybridizations were performed on genomic DNA from both *A. thaliana* and *H. schachtii* with each cDNA used as a probe (data not shown). Hybridization signals showed that 24 cDNA clones were of *A. thaliana* ori-

gin while 12 clones stemmed from *H. schachtii* (Tables 1 and 2). Judging from the differential display analyses, of the 24 *A. thaliana* cDNA clones, 13 corresponded to mRNA species that were more abundant in the infected root sections and the remaining 11 corresponded to mRNA species that were more abundant in the uninfected root sections. Similarly, of the 12 *H. schachtii* cDNA clones, four corresponded to mRNA species that were more abundant in the parasitic nematode and the remaining eight corresponded to mRNA species that were more abundant in the preparasitic nematode. The hybridization signal intensity differences between duplicate slot blots of cDNA clones probed with the respective differential display reaction mixes (see Materials and Methods) were used as a crude measure to quantify mRNA abundance changes between infected and uninfected root sections (Tables 1 and 2).

Sequence comparisons.

BLAST data base searches (Table 1) revealed strong similarities to previously identified proteins for 10 *A. thaliana* cDNA sequences (A6.1; A10.1; A19.5; A20.2; A24.4; A28.5; A30.1; A36.2; A43.2; A44.3). For 14 *A. thaliana* cDNA sequences no putative protein identity could be ascribed. These latter sequences were similar to *A. thaliana* expressed sequence tags or genomic sequences or did not show any similarity to available data base entries. BLAST data base searches querying the 12 *H. schachtii* cDNA sequences (Table 2) revealed strong similarities to known proteins for three cDNAs (A22.1; A27.3; A53.1).

In situ hybridization analysis.

Because of the rare starting material used for the differential display experiments, RNA gel blot analyses to confirm the

Table 1. Initial characterization of *Arabidopsis thaliana* cDNA clones

Clone name	GenBank accession ^a	mRNA change ^b	Protein similarity ^c	BLAST score	BLAST E value
A1.1	AI618740	-6	None		
A6.1	AI618741	+210	<i>Medicago sativa</i> isocitrate dehydrogenase	48	0.001
A9.4	AI618742	-12	None		
A10.1	AI618743	-72	<i>Brassica oleracea</i> putative major latex protein.	60	5×10^{-7}
A13.2	AI618745	-20	None		
A15.4	AI618746	+237	None		
A16.2	AI618747	-10	None		
A17.6	AI618748	+68	None		
A19.5	AI618749	+171	<i>Beta vulgaris</i> glucose-1-phosphate adenylyltransferase	67	3×10^{-11}
A20.2	AI618750	-59	<i>B. oleracea</i> putative major latex protein	60	4×10^{-7}
A24.4	AI618751	+68	<i>A. thaliana</i> putative inositol polyphosphate 5-phosphatase	153	8×10^{-36}
A28.5	AI618753	+68	<i>A. thaliana</i> actin-12	62	4×10^{-8}
A30.1	AI618754	-26	<i>A. thaliana</i> AP2 domain containing protein RAP2.3	270	1×10^{-70}
A31.4	AI618755	-62	None		
A32.3	AI618756	+65	None		
A36.2	AI618757	-13	<i>A. thaliana</i> calmodulin-domain protein kinase	167	5×10^{-40}
A37.3	AI618758	-85	None		
A38.3	AI618759	+113	None		
A41.3	AI618761	+102	None		
A42.2	AI618762	+240	None		
A43.2	AI618763	+80	<i>A. thaliana</i> putative lysophospholipase.	62	6×10^{-10}
A44.3	AI618764	+7	<i>A. thaliana</i> NADH:ubiquinone oxidoreductase	385	1×10^{-105}
A46.4	AI618765	+31	None		
A55.3	AI618766	-65	None		

^a Sequences were entered also into dbEST.

^b Negative values indicate lower mRNA abundance and positive values indicate higher mRNA abundance in infected versus uninfected root sections. Values were calculated by expressing the difference between slot blot hybridization signals (see Materials and Methods for explanation of slot blots) obtained for infected and uninfected root section pools as a percentage of the hybridization signal obtained for the uninfected root section pool.

^c Putative identifications were made with BLASTN or BLASTX searches. Weak similarities and identities with putative proteins or "expressed sequence tags" are not shown.

mRNA abundance changes predicted by the differential display results are difficult to perform. We, nevertheless, compared mRNA abundance of all 24 *A. thaliana* cDNA clones on blots of total RNA from whole infected and uninfected roots. Only five transcripts were detectable, and none of these showed significant differences between infected and uninfected roots (data not shown). These results suggest that local differences in transcript abundance at the developing syncytium are too subtle for detection when infected and uninfected whole roots are compared. More sensitive in situ hybridization analyses were subsequently performed to scrutinize differences in mRNA accumulation indicated by differential display analyses.

For these experiments we chose one *A. thaliana* cDNA clone that corresponds to an mRNA with a predicted decrease in infected root sections (A30.1) and one cDNA clone that corresponds to an mRNA predicted to increase in infected root sections (A42.2) (Table 1). mRNA for both clones was weakly detectable on total RNA blots (data not shown). A30.1 is identical to a sequence at the 3' end of the *A. thaliana* RAP2.3 mRNA, and A42.2 did not have a convincing similarity in available data bases at the time of analysis. Three days after inoculation with *H. schachtii*, infected *A. thaliana* roots were harvested, fixed, and embedded in paraffin blocks. Sections of the blocks were affixed sequentially onto numbered slides and hybridized with either sense (negative control) or antisense digoxigenin (DIG)-labeled RNA probes derived from the two cDNA clones. Hybridized probe was visualized by alkaline phosphatase-mediated color reaction as described in Materials and Methods. Slides were then assessed for hybridization signals. Control slides probed with sense strands did not show any signal in *A. thaliana* root tissue, although nonspecific signal slightly stained the nematode (Fig. 1A and C). Tissue sections hybridized with the antisense probe derived from A30.1 confirmed that A30.1 mRNA was less abundant in the area of the syncytium than in adjacent nematode-free areas (Fig. 1B). Additionally, tissue sections hybridized with the antisense probe corresponding to A42.2 confirmed that A42.2 mRNA was detectable in the area of the syncytium but not in adjacent areas (Fig. 1D). Both these results were confirmed in several independent root pieces and in consecutive sections of each

root piece tested. These findings lend strong support to the robustness of our differential display data. Antisense probes from both clones (A30.1 and A42.2) also showed strong staining in lateral root buds (Fig. 1E, F) and tips of lateral roots (Fig. 1H, only A30.1 shown).

DISCUSSION

The small dimensions of nematode syncytia and their mosaic-like distribution within host roots lead to a low ratio of cells involved in syncytium formation to those that are not. When gene expression in *H. schachtii*-infected whole roots is compared with that in uninfected whole roots, this low ratio renders the identification of gene expression changes associated with developing syncytia difficult. Therefore, we devised a sampling strategy that should allow the identification of mRNA abundance changes in the vicinity of the developing syncytium. This same sampling strategy should eliminate the detection of systemic mRNA abundance changes that are observable in nematode-infected roots, because those mRNAs should have been present in both root samples analyzed in our study. Taking these assumptions into account, one would expect that mRNAs identified in our study (i) may be of low abundance in whole roots and (ii) should not necessarily be of different abundance in infected versus uninfected whole roots. These expectations were supported by our Northern (RNA) blot analyses.

Our sampling strategy also provided another advantage. The 1:1 ratio of nematodes to plant sections in our sample pools allowed the identification of nematode mRNA species whose abundance change during the transition from preparasitic (i.e., before root penetration) to parasitic (i.e., after root penetration) life style. In our study, one third of the cDNA clones isolated were of *H. schachtii* origin (Table 2). In a previously conducted differential display project in which mRNA changes were assayed in whole soybean root-explants infected by the soybean-cyst nematode, *H. glycines*, no cDNA clones of nematode origin were identified (Hermsmeier et al. 1998). These observations again indicate the high quality of starting material chosen for this study. Further characterization of the *H. schachtii* mRNAs and their corresponding genes also is of

Table 2. Initial characterization of *Heterodera schachtii* cDNA clones

Clone name	GenBank accession ^a	mRNA change ^b	Protein similarity ^c	BLAST score	BLAST E value
A12.2	AI618767	-36	None		
A14.4	AI618768	-90	None		
A18.3	AI618769	-72	None		
A22.1	AI618770	-58	<i>Caenorhabditis elegans</i> putative 60s ribosomal protein	74	3×10^{-13}
A27.3	AI618771	-39	<i>Drosophila melanogaster</i> acidic ribosomal protein	101	2×10^{-21}
A45.1	AI618773	-53	None		
A46.2	AI618774	+1167	None		
A48.2	AI618775	+149	None		
A51.2	AI618777	+248	None		
A53.1	AI618778	-58	<i>Homo sapiens</i> vacuolar protein	98	9×10^{-21}
A54.1	AI618779	-76	None		
A58.4	AI618780	+74	None		

^a Sequences were entered also into dbEST.

^b Negative values indicate lower mRNA abundance and positive values indicate higher mRNA abundance in parasitic versus preparasitic J2 nematodes. Values were calculated by expressing the difference between slot blot hybridization signals (see Materials and Methods for explanation of slot blots) obtained for infected and uninfected root section pools as a percentage of the hybridization signal obtained for the uninfected root section pool.

^c Putative identifications were made with BLASTN or BLASTX searches. Weak similarities and identities with putative proteins or "expressed sequence tags" are not shown.

Fig. 1. In situ hybridization with digoxigenin (DIG)-labeled riboprobes corresponding to clones A30.1 or A42.2 in *Heterodera schachtii*-infected *Arabidopsis thaliana* roots. Accumulation of mRNA is indicated by brownish-purple staining. Hybridization of (A) sense and (B) antisense probes derived from A30.1 in longitudinal root sections harboring a nematode (n). Hybridization of (C) sense and (D) antisense probes derived from A42.2 in longitudinal root sections harboring a nematode (n). Hybridization of antisense probes derived from (E) A30.1 and (F) A42.2 in lateral root buds (lr). Hybridization of (G) sense and (H) antisenses probes derived from A30.1 in cross sections of the meristematic region of lateral root tips. Staining similar to that seen in H was obtained for the A42.2 antisense probe (data not shown). Size bar = 100 μ m in A–F and 10 μ m in G and H.

promise to elucidate the interactions between nematode and plant. In fact, the comparison of mRNA abundance in parasitic versus parasitic root-knot nematodes (*Meloidogyne incognita*) identified a putative parasitism gene coding for a protein likely to be injected through the nematode stylet into root tissues of parasitized plants (Ding et al. 1998). The *H. schachtii* cDNA clones presented in this paper will be the subject of research efforts to identify their site and timing of mRNA accumulation via in situ hybridization.

Microbial contaminants carried by the invading nematodes pose potential obstacles in studies like the one presented in this paper. Our use of monoxenic culturing conditions eliminated the possibility of accidentally identifying cDNAs of microbial origin or from plant genes responding to contaminants. Therefore, we have a high level of confidence that the observed mRNA abundance changes are due to nematode penetration and/or infection alone. Furthermore, the in situ hybridizations for two of the identified cDNA clones confirm the mRNA abundance changes predicted by the differential display analyses. We, therefore, conclude that the remaining cDNA clones likely behave in vivo as predicted by the differential display analyses.

One of the cDNAs assayed by in situ hybridization, A30.1, is identical to a region of the 3' untranslated region (UTR) of the *A. thaliana* RAP2.3 mRNA (accession number AF003096; Okumuro et al. 1997). RAP2.3 is a member of the AP2 domain DNA-binding protein family. Judging by the number of AP2 domains and other conserved regions contained in this protein, RAP2.3 belongs to the family of ethylene-responsive element binding proteins (EREBPs; Okumuro et al. 1997). Furthermore, RAP2.3 is 99% identical to the translation product of the *A. thaliana* ethylene-responsive element binding protein mRNA (AtEBP; accession number Y09942). This mRNA is induced by ethylene, and the AtEBP binds to the GCC box that confers ethylene responsiveness to a number of pathogenesis-related (PR) gene promoters (Büttner and Singh 1997). A direct function of EREBPs in activating the defense response mediated by the *Pto* resistance gene has been shown in tomato (Zhou et al. 1997). Our in situ hybridization results reveal that the mRNA corresponding to A30.1 accumulates systemically in roots (Fig. 1B and E), but is locally down-regulated at the syncytium. The latter conclusion also can be drawn from the differential display results. We hypothesize that RAP2.3 is a putative positive regulator of plant defense response genes that is locally down-regulated in response to the nematode as a means to avoid plant defenses. This hypothesis provides a good starting point for future research on the involvement of EREBPs in plant-nematode interactions. Such studies could be initiated by directing transcription of the RAP2.3 mRNA to developing syncytia in transgenic plants with the promoter of A42.2 (see below) or other cyst nematode-responsive regulatory regions reported in the literature (Barthels et al. 1997).

The other clone used for in situ hybridization (A42.2) also supported the differential display results. mRNA corresponding to clone A42.2 was shown to accumulate preferentially in the region of the developing syncytium (Fig. 1D). The obtained hybridization signal is much weaker in adjacent, nematode-free sections of the root. Furthermore, in Figure 1F it is apparent that, unlike A30.1 (Fig. 1E), A42.2 mRNA does not accumulate visibly in uninfected root tissue except the lat-

eral root bud and root tips. We hypothesize that the gene corresponding to A42.2 may have a function in or around the developing syncytium. Because of this differential pattern of mRNA accumulation, regulatory regions of the gene corresponding to A42.2 may be of use to direct transcription of genes into the developing *H. schachtii* syncytium in efforts to devise anti-nematode strategies.

mRNA species corresponding to A30.1 and A42.2 were shown via in situ hybridization to accumulate in lateral root buds (Fig. 1E, F) and root tips (Fig. 1H, only A30.1 shown). Lateral roots arise from the pericycle of roots but are otherwise similar to primary root tips (Dolan et al. 1993). Lateral root buds and root tips harbor small, cytoplasmically dense, and metabolically active cells. Lateral root buds and syncytia share several aspects in common: both can arise from pericycle cells and both require a change in differentiation to a more cytoplasmically dense and metabolically active state. Barthels et al. (1997) showed that a large proportion of promoters that respond to nematode infection also direct reporter gene expression to lateral root buds. Additionally, Niebel et al. (1996) showed that the cell division competence marker *cdc2a* is expressed in both syncytia and lateral root meristems. Consequently, several genes involved in lateral root initiation may also have functions in syncytium formation or function. Our preliminary characterization of A42.2 is in accordance with this notion.

The main goal of this differential display project was to identify plant mRNAs that change locally at the developing nematode syncytium. Such *A. thaliana* cDNAs are good candidates for further study, because they may include plant genes activated by the nematode to aid in syncytium formation. On the other hand, we may find plant genes deactivated by the nematode to allow syncytium formation to proceed. The identities and functions of some of the identified cDNA clones will be the starting points to formulate testable hypotheses to further explore the *H. schachtii* - *A. thaliana* interaction. The identification of 24 *A. thaliana* genes that are predicted to be locally up- or down-regulated at the syncytium plus the identification of 12 *H. schachtii* genes that are predicted to change expression during the transition to parasitism mark a promising beginning for the study of both plant and nematode genes involved in the compatible interaction.

MATERIALS AND METHODS

Plant and nematode material.

A. thaliana seeds (ecotype Columbia; Lehle Seeds, Round Rock, TX), surface sterilized in 50% commercial bleach for 5 min, were germinated in petri dishes containing 0.8% Daishin agar (Brunschwig Chemie, Amsterdam, The Netherlands) supplemented with Knop medium as described by Sijmons et al. (1991). Seedlings grown at 25°C under 10 W/m² of fluorescent light with a photoperiod of 12 h were inoculated with J2 of *H. schachtii* 7 to 10 days post germination. A *H. schachtii* field population designated TN101 (kindly provided by G. Tylka, Iowa State University, and originally obtained from T. Niblack, University of Missouri), was grown in greenhouse cultures on cabbage or sugarbeet plants. This nematode culture had previously been cultured for 10 years on cabbage and can be considered inbred by mass selection. Infective nematodes were collected and surface sterilized as de-

scribed in Hermsmeier et al. (1998). Two hundred J2, suspended in 20 µl of 1.5% low-melting point agarose (Life Technologies, Gaithersburg, MD) held at 37°C, were used to inoculate individual seedlings. Two to 3 days post inoculation, infected root samples and control tissue were harvested. For differential display experiments, samples consisted of syncytia and attached worms, while control tissue consisted of adjacent, nematode-free tissue to which an equal number of preparasitic J2 was added. For Northern blots, samples consisted of whole, infected roots or their shoots, and control tissue consisted of whole, uninfected roots or their shoots. For in situ hybridization, samples consisted of whole, infected roots.

Differential display, slot blots, and sequence analysis.

Total RNA was extracted from infected and uninfected root sections as described in Pawlowski et al. (1994). mRNA was reverse transcribed with oligo-T primers, and first-strand cDNA pools derived from either infected or uninfected root sections were used as templates for differential display reactions with ³³P as isotope label. Differential display reactions, slot blots, autoradiography, and phosphorimage analysis were performed as described previously (Hermsmeier et al. 1998). In short, differential display used the selective anchorprimers 5'-T₁₂AC-3', 5'-T₁₂CA-3', 5'-T₁₂CC-3', 5'-T₁₂GA-3', 5'-T₁₂GC-3', and 5'-T₁₂GG-3'. Sequences of arbitrary primers were 5'-GGAACCAATC-3' (R3), 5'-TCGGTCATAG-3' (R5), 5'-GGTACTAAGG-3' (R6), 5'-CTGCTTGATG-3' (R7), 5'-GATCACGTAC-3' (R8), and 5'-GATCAATCGC-3' (R9). Radiolabeled, differential display amplification products were separated on sequencing-type gels, and the gels were dried and subjected to autoradiography. Autoradiographs were surveyed for signal intensity differences between bands derived from the infected and uninfected root sections. Gel regions corresponding to bands that exhibited intensity differences were excised, and the DNAs in these gel pieces were eluted, re-amplified by polymerase chain reactions, ligated into a plasmid cloning vector, and transformed into *Escherichia coli*. Recombinant plasmid DNAs were isolated from six colonies per transformation, and the DNAs were slot blotted in duplicate. The slot blots were then probed with the differential display reaction mixes from either infected or uninfected root sections. Quantification, with a phosphorimager, of hybridization intensity differences between duplicate slot blots allowed the identification of recombinant plasmids harboring the cDNA fragment that gave rise to the polymorphism detected in the differential display gel (Vögeli-Lange et al. 1996). Clones that duplicated the intensity differences on the differential display autoradiograph were retained for further analysis. Nucleotide sequences were determined at the Iowa State University DNA Analyses and Synthesis Facility, and analyses were performed with the BLAST program (Altschul et al. 1997).

In situ hybridization.

Infected root tissue was fixed in 2.5% glutaraldehyde, 0.1 M cacodylate buffer pH 7.2 and incubated at room temperature under sequentially increasing and decreasing vacuum pressure for 2 h. Tissue was then immersed in fresh fixative and incubated overnight at 4°C on a specimen rotator. Tissue samples were sequentially dehydrated 10 min each in 30, 50,

70, and 95% ethanol, three times for 15 min each in 100% ethanol, sequentially in xylene:ethanol 25:75, 50:50, 75:25, and three times for 15 min each in 100% xylene. Tissue samples, 20 to 30 roots per block, were then embedded in paraplast over 3 days. Embedded tissue samples were sectioned 8 µm thick on a rotary microtome. Sections were floated on deionized water, sequentially ordered on numbered poly-L lysine-coated microscope slides, and fixed to the slides with a 42°C warming tray overnight. Slides with attached tissue sections were deparaffinized with xylene and hydrated through an ethanol series to 50 mM TE (Tris-EDTA) buffer pH 7.6, treated with 1 µg of proteinase K per ml in 50 mM TE buffer pH 7.6 for 20 min, washed, treated with 0.25% acetic anhydride in 85 mM triethanolamine buffer pH 8.0 for 5 min, washed, and dehydrated through an ethanol series to dry completely. Hybridization took place in a humidified chamber at 45°C with 0.6 µg of DIG-labeled RNA probe per ml in 50% formamide overnight. The hybridization solution also contained 300 mM NaCl, 10 mM Tris pH 7.5, 1 mM 0.5 M EDTA, 1× Denhardt's solution, 10% dextran sulfate, 10 mM DTT (dithiothreitol), 250 ng of tRNA per µl, 100 µg of poly-A per ml, and DEPC (diethyl pyrocarbonate) water to volume.

Sense and antisense riboprobes corresponding to clones A30.1 and A42.2 were transcribed with T7 and T3 polymerases, and were purified from the template with DNase I. Unincorporated nucleotides were removed by column purification, and incorporation of DIG-UTP was quantified by a dot blot procedure. Probes were only 189 and 143 bp in length (A30.1 and A42.2, respectively), so hydrolysis of probes was not necessary.

After hybridization, slides were washed 15 min in 2× SSC at 25°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), three times for 25 min each in 0.2× SSC at 55°C, treated with 20 µg of RNase A per ml in 500 mM NaCl/TE buffer pH 8.0 at 37°C for 30 min, washed three times in sterile, deionized water, incubated in buffer 1 (1% blocking solution, 100 mM Tris pH 7.5, 150 mM NaCl) for 1 h, then equilibrated with buffer 2 (100 mM Tris pH 7.5, 150 mM NaCl, 0.5% BSA [bovine serum albumin], and 0.3% Triton X-100). Tissue sections were then incubated with an alkaline-phosphatase conjugated antibody to DIG, diluted 1:1000 in buffer 2 in a humidified chamber for 2 h, then washed three times for 20 min each in 100 mM Tris pH 7.5, 150 mM NaCl. The tissue sections were equilibrated in buffer 3 (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 10 min, then incubated in 3.2 µg of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) per ml: 6.6 µg of nitro-blue tetrazolium salt (NBT) per ml in buffer 3 in a humidified chamber in the dark for 4 h. Slides were then dehydrated through an ethanol series and dried overnight. Coverslips were affixed with Permount (Fisher Scientific, Pittsburgh, PA) to make permanent slides. Each slide was scanned for root sections containing a worm, a developing syncytium, and adjacent uninfected tissue. Each slide also was scanned for other distinguishing patterns of accumulation. Representative sections were documented with an Axiophot Photomicroscope (Carl Zeiss, Thornwood, NY) and a Sony DXC-3000A color video camera (Sony Electronics, Park Ridge, NJ). Images were digitally captured with the SGI O2 computer program (Silicon Graphics, Mountain View, CA) and reformatted for publication with Adobe Photoshop (Adobe Systems, San Jose, CA).

ACKNOWLEDGMENTS

This is Journal Paper No. J-18423 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3502, and supported by Hatch Act and State of Iowa funds. Funding also was provided by USDA-NRICGP Award # 97-35302-4866, the Iowa Soybean Promotion Board, and the Iowa State University Carver Trust Fund and Biotechnology Council. We thank T. R. Maier, J. M. de Boer, E. Skinner, and the staff at the Iowa State University Microscope and Image Analysis facilities for technical assistance. We also thank J. M. de Boer and A. Schwekendiek for critical review of the manuscript.

LITERATURE CITED

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Atkinson, H. J. 1995. Pathogenesis and host specificity in plant diseases. Pages 355-370 in: *Histopathological, Biochemical, Genetic and Molecular Bases of Diseases*. Vol. 2: Eukaryotes. K. Kohmoto, U. S. Singh, and R. P. Singh, eds. Pergamon Press, Elsevier, Oxford.
- Barthels, N., van der Lee, F. M., Klap, J., Goddijn, O. J., Karimi, M., Puzio, P., Grundler, F. M., Ohl, S. A., Lindsey, K., Robertson, L., Robertson, W. M., Van Montagu, M., Gheysen, G., and Sijmons, P. C. 1997. Regulatory sequences of *Arabidopsis* drive reporter gene expression in nematode feeding structures. *Plant Cell* 9:2119-34.
- Büttner, M., and Singh, K. B. 1997. *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. *Proc. Natl. Acad. Sci. USA* 94:5961-5966.
- de Boer, J. M., Yan, Y., Wang, X., Smant, G., Hussey, R. S., Davis, E. L., and Baum, T. J. 1999. Developmental expression of secretory β -1,4-endoglucanases in the subventral esophageal glands of *Heterodera glycines*. *Mol. Plant-Microbe Interact.* 12:663-669.
- Ding, X., Shields, J., Allen, R., and Hussey, R. S. 1998. A secretory cellulose-binding protein cDNA cloned from the root-knot nematode (*Meloidogyne incognita*). *Mol. Plant-Microbe Interact.* 11:952-959.
- Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. 1993. Cellular organisation of the *Arabidopsis thaliana* root. *Development* 119:71-84.
- Favery, B., Lecomte, P., Dil, N., Bechtold, N., Bouchez, D., Dalmaso, A., and Abad, P. 1998. *RPE*, a plant gene involved in early developmental steps of nematode feeding cells. *EMBO J.* 17:6799-6811.
- Goddijn, O. J., Lindsey, K., van der Lee, F. M., Klap, J. C., and Sijmons, P. C. 1993. Differential gene expression in nematode-induced feeding structures of transgenic plants harbouring promoter-gusA fusion constructs. *Plant J.* 4:863-73.
- Golinowski, W., Grundler, F. M. W., and Sobczak, M. 1996. Changes in the structure of *Arabidopsis thaliana* during female development of the plant-parasitic nematode *Heterodera schachtii*. *Protoplasma* 194: 103-116.
- Goverse, A., Smant, G., Gommers, F. J., Helder, J., Schots, A., and Bakker, J. 1998. In planta monitoring of the activity of two constitutive promoters, CaMV 35S and TR2, in developing feeding cells induced by *Globodera rostochiensis* using GFP in combination with confocal laser scanning microscopy. *Physiol. Mol. Plant Pathol.* 52:275-284.
- Gurr, S. J., McPherson, M. J., Scollan, C., Atkinson, H. J., and Bowles, D. J. 1991. Gene expression in nematode-infected plant roots. *Mol. Gen. Genet.* 226:361-366.
- Hermesmeier, D., Mazarei, M., and Baum, T. J. 1998. Differential display analysis of the early compatible interaction between soybean and the soybean cyst nematode. *Mol. Plant-Microbe Interact.* 11:1258-1263.
- Hussey, R. S. 1989. Disease-inducing secretions of plant-parasitic nematodes. *Annu. Rev. Phytopathol.* 27:123-141.
- Liang, P., and Pardee, A. B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967-971.
- Møller, S. G., Urwin, P. E., Atkinson, H. J., and McPherson, M. J. 1998. Nematode-induced expression of *atao1*, a gene encoding an extracellular diamine oxidase associated with developing vascular tissue. *Physiol. Mol. Plant Pathol.* 53:73-79.
- Niebel, A., de Almeida Engler, J., Hemerly, A., Ferreira, P., Inze, D., Van Montagu, M., and Gheysen, G. 1996. Induction of *cdc2a* and *cyc1At* expression in *Arabidopsis thaliana* during early phases of nematode-induced feeding cell formation. *Plant J.* 10:1037-43.
- Okamura, J. K., Caster, B., Villarroel, R., Van Montagu, M., and Jofuku, D. D. 1997. The AP2 domain of *APETALA2* defines a large new family of DNA binding proteins in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 94:7076-7081.
- Pawlowski, K., Kunze, R., De Vries, S., and Bisseling, T. 1994. Isolation of total, poly(A) and polysomal RNA from plant tissues. Pages D5/1-D5/4 in: *Plant Molecular Biology Manual*. S. B. Gelvin and R. A. Schilperoort, eds. Kluwer Academic, Dordrecht, The Netherlands.
- Puzio, P. S., Cai, D., Ohl, S., Wyss, U., and Grundler, F. M. W. 1998. Isolation of regulatory DNA regions related to differentiation of nematode feeding structures in *Arabidopsis thaliana*. *Physiol. Mol. Plant Pathol.* 53:177-193.
- Sasser, J. N., and Freckman, D. W. 1987. A world perspective on nematology: The role of the society. Pages 7-14 in: *Vistas on Nematology*. J. A. Veech and D. W. Dickerson, eds. Society of Nematologists, Hyattsville, MD.
- Sijmons, P. C., Atkinson, H. J., and Wyss, U. 1994. Parasitic strategies of root nematodes and associated host cell responses. *Annu. Rev. Phytopathol.* 32:235-259.
- Sijmons, P. C., Grundler, F. M. W., von Mende, N., Burrows, P. R., and Wyss, U. 1991. *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *Plant J.* 1:245-254.
- Smant, G., Stokkermans, J. P. W. G., Yang, Y., de Boer, J. M., Baum, T. J., Wang, X., Hussey, R. S., Gommers, F. J., Henrissat, B., Davis, E. L., Helder, J., Schots, A., and Bakker, J. 1998. Endogenous cellulases in animals: Isolation of β -1,4-endoglucanases from two species of plant-parasitic nematodes. *Proc. Natl. Acad. Sci. USA* 95:4906-4911.
- Vögeli-Lange, R., Brückert, N., Boller, T., and Wiemken, A. 1996. Rapid selection and classification of positive clones generated by mRNA differential display. *Nucleic Acids Res.* 24:1385-1386.
- Wang, X., Meyers, D., Yan, Y., Baum, T., Smant, G., Hussey, R., and Davis, E. 1999. In planta localization of a β -1,4-endoglucanase secreted by *Heterodera glycines*. *Mol. Plant-Microbe Interact.* 12:64-67.
- Williamson, V. M., and Hussey, R. S. 1996. Nematode pathogenesis and resistance in plants. *Plant Cell* 8:1735-1745.
- Wrather, J. A., Anderson, T. R., Arsyad, D. M., Gai, J., Ploper, L. D., Porta-Puglia, A., Ram, H. H., and Yorinori, J. T. 1997. Soybean disease loss estimates for the top 10 soybean producing countries in 1994. *Plant Dis.* 81:107-110.
- Zhou, J. M., Tang, X. Y., and Martin, G. B. 1997. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *EMBO J.* 16:3207-3218.