

# Characterization of *LeMir*, a Root-Knot Nematode-Induced Gene in Tomato with an Encoded Product Secreted from the Root<sup>1</sup>

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A tomato gene that is induced early after infection of tomato (*Lycopersicon esculentum* Mill.) with root-knot nematodes (*Meloidogyne javanica*) encodes a protein with 54% amino acid identity to miraculin, a flavorless protein that causes sour substances to be perceived as sweet. This gene was therefore named *LeMir* (*L. esculentum* miraculin). Sequence similarity places the encoded protein in the soybean trypsin-inhibitor family (Kunitz). *LeMir* mRNA is found in root, hypocotyl, and flower tissues, with the highest expression in the root. Rapid induction of expression upon nematode infection is localized to root tips. In situ hybridization shows that *LeMir* is expressed constitutively in the root-cap and root-tip epidermis. The *LeMir* protein product (LeMir) was produced in the yeast *Pichia pastoris* for generation of antibodies. Western-blot analysis showed that LeMir expression is up-regulated by nematode infection and by wounding. LeMir is also expressed in tomato callus tissue. Immunoprint analysis revealed that LeMir is expressed throughout the seedling root, but that levels are highest at the root/shoot junction. Analysis of seedling root exudates revealed that LeMir is secreted from the root into the surrounding environment, suggesting that it may interact with soil-borne microorganisms.

Root-knot nematodes (*Meloidogyne* spp.) are endoparasites of the roots of most cultivated crops and cause significant economic losses worldwide (Sasser, 1980). This group of nematodes has a complex life cycle (Williamson and Hussey, 1996). The infective stage, the second-stage juvenile, is attracted to root tips, where it penetrates the zone of elongation and then migrates intercellularly, first toward the root tip and then up to the developing vascular tissue (Wyss et al., 1992). There the nematode initiates a feeding site, causing the formation of large, multinucleate, metabolically active giant cells (Jones, 1978; Huang, 1985). Nearby cells of the cortex, pericycle, and vascular parenchyma enlarge and divide, forming a root-knot or gall. Initiation of the gall can be seen under the microscope

between 12 and 24 h after inoculation. Although tomato (*Lycopersicon esculentum* Mill.) is an excellent host for these nematodes, some varieties are resistant because of the presence of the dominant gene *Mi*. The presence of *Mi* is correlated with the development of a localized necrosis of host cells at the feeding site within 24 h of infection (Dropkin et al., 1969; Paulson and Webster, 1972).

Because of the economic importance of the root-knot nematode, the molecular biology of the formation and maintenance of the feeding site has been studied by a number of authors (for review, see Williamson and Hussey, 1996). Several genes have been characterized that are up-regulated in the giant cell and the gall (Goddijn et al., 1993; Niebel et al., 1993, 1995, 1996; Bird and Wilson, 1994; Opperman et al., 1994). However, very little is understood regarding molecular changes that occur in the root early after infection, before giant-cell initiation or induction of the hypersensitive response. Genes induced early after nematode infection could potentially have a role in the defense against nematodes or other root pathogens. Overall, root defense systems are poorly understood compared with shoot systems. Of the cases examined, proteins induced in the root during pathogenesis are similar to antimicrobial proteins found in the shoot, such as chitinase,  $\beta$ -1,3-glucanase, osmotin, and ribosome-inactivating protein (Maraganore et al., 1987; Benhamou et al., 1990, 1993; Neale et al., 1990; Savary and Flores, 1994; Savary et al., 1997).

To identify rapidly up-regulated, nematode-induced plant genes with a possible role in defense against nematodes or other root parasites, we developed a technique to obtain synchronously infected root tips and then produced a cDNA library from them (Ho et al., 1992; Lambert and Williamson, 1993). Several genes that are increased in expression by 12 h after nematode infection were identified by differential screening (Williamson et al., 1994; Lambert, 1995). Most of these genes appeared to be equally induced in plants independent of the presence of *Mi* in the genome. Some have homology to known plant defense genes, including those coding for peroxidase, chitinase, lipoxygenase, and proteinase inhibitors (Lambert, 1995; B. Ferrie and V.M. Williamson, unpublished data). One nematode-induced cDNA, clone 23a, encoded the partial sequence of a protein with high similarity to that of miraculin, a protein

<sup>1</sup> This work was supported by grants from the U.S. Department of Agriculture-National Research Initiative Competitive Grants Program (nos. 92-37302-7635 and 94-37302-0569).

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Abbreviations: EF, elongation factor; RACE, rapid amplification of cDNA ends.

isolated from the berries of *Richadella dulcifica*, a west-African shrub.

Miraculin alters human taste perception, converting sour into sweet taste (Theerasilp et al., 1989). Because of its sequence similarity to miraculin, the tomato gene was named *LeMir* (*L. esculentum* *miraculin*). The sequence also shows similarity to the soybean trypsin-inhibitor family. Several members of this family have anti-insect/anti-pathogen activity (Ryan, 1990), suggesting that *LeMir* may have a role in defense against nematodes or other pathogens/pests. Furthermore, *LeMir* shows very high similarity to TID91, a gene of unknown function that is highly expressed in tobacco genetic tumors (Fujita et al., 1994). In the present study, we characterized the *LeMir* cDNA sequence and we present information regarding its expression pattern at the mRNA and protein levels.

## MATERIALS AND METHODS

### Plant Material and Nematode Infection

Seeds of the tomato (*Lycopersicon esculentum* Mill.) cvs VFN8 and VFNT cherry were obtained from Petoseed (Woodland, CA) and from C. Rick (University of California, Davis), respectively.

One-day-old root-knot nematodes (*Meloidogyne javanica* strain VW4 second-stage juveniles) were collected from hydroponic cultures (Lambert et al., 1992) and used to infect 5-d-old tomato seedlings on filter paper (Ho et al., 1992; Lambert, 1995).

### Nucleic Acid Sequencing

The nucleic acid sequence of cDNA 23a was determined using a DNA-sequencing kit (Sequenase, United States Biochemical) (Sanger et al., 1977). The DNA sequences of the 5' end of the *LeMir* cDNA and the genomic clone were determined by automated sequencing carried out by the Iowa State University DNA Sequencing and Synthesis Facility (Ames). Computer analysis of DNA sequence data was performed using the software package from the Genetics Computer Group (Madison, WI). Sequence similarities were identified using BLASTX (Gish and States, 1993).

### Modified RACE-PCR

The 5' end of the *LeMir* cDNA was cloned using a modification of the RACE procedure of Frohman et al. (1988). Total RNA was isolated from 50 cv VFN8 root tips infested with root-knot nematodes for 12 h. Poly(A<sup>+</sup>) RNA was hybridized to Dynabeads oligo(dT)<sub>25</sub> (Dynal, Great Neck, NY). First- and second-strand cDNA strands were synthesized on the beads as described previously (Lambert and Williamson, 1993). The beads were washed three times in PCR buffer, and the second-strand cDNA was eluted into 50  $\mu$ L of water; 5  $\mu$ L of this solution was amplified in a 50- $\mu$ L reaction containing PCR buffer, 25 pmol of *LeMir* antisense primer 23a1 (5'-TGAAGACTTGACTAGCCT-3'), and 25 pmol of L-primer (Lambert and Williamson, 1993) for 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for

1 min. The amplified products were electrophoresed on a 2% agarose gel and the correct-sized band was cut from the gel, purified, and resuspended in 20  $\mu$ L of water. The DNA was diluted 10-fold with water and 1  $\mu$ L of the diluted cDNA was amplified using L-primer and primer 23a3 (5'-CGTCTAACTCCATCCTGAAT-3'), an antisense primer 50 bp 5' to primer 23a1. The products were purified and cloned into pCRII using a kit (TA, Invitrogen, San Diego, CA).

### Isolation of a Genomic Clone of *LeMir*

A phage genomic DNA library made from cv VFNT DNA (a gift of R. Fischer, University of California, Berkeley) was screened using clone 23a as a probe (Sambrook et al., 1989). A hybridizing clone was detected, and from it a 3.32-kb *Eco*RI fragment containing the full-length *LeMir*-coding region, as well as 1.18-kb untranscribed upstream and 1.32-kb downstream DNA, were subcloned into pBlue-script II SK. The resulting plasmid was called pEB3.

### Creation of a *LeMir* Subclone Truncated at the N Terminus

A subclone containing a portion of the *LeMir*-coding region was made from the plasmid pEB3. A PCR fragment was generated starting at bp 73 downstream from the ATG translation start site and ending at the translation termination stop codon TAG. The primers were designed to produce flanking *Eco*RI sites at the ends of the amplified PCR fragment. The fragment was cloned into the plasmid pCRII. Subclones in both orientations were sequenced. The subclones that produced sense and antisense products were named pEB28 and pEB29, respectively.

### Northern-Blot Analysis

RNA was isolated as described by Rochester et al. (1986), resuspended in diethyl pyrocarbonate-treated water denatured using glyoxal/DMSO, and electrophoresed on a 2% agarose gel in phosphate buffer (Sambrook et al., 1989). The RNA was then transferred to a nylon membrane and fixed to the membrane by UV cross-linking. Blots were prehybridized and hybridized using standard protocols (Sambrook et al., 1989) and labeled 23a as a probe. The final wash was in 0.1 $\times$  SSC, 0.1% SDS at 65°C.

### Quantification of Autoradiographic Signals

Quantification of autoradiographic signals was carried out using an image-analysis system consisting of a CCD video camera (Sanyo) fitted with a 75-mm zoom lens linked to a computer (model 386/25, Everex, Fremont, CA) via a video-capture board (Targa Plus, Truevision, Indianapolis, IN). Images were captured using the Java software package (Jandel Scientific, San Rafael, CA). The captured images were analyzed with imaging software from the National Institutes of Health (Bethesda, MD). In all quantified autoradiographs the cDNA signals were normalized to a cyclophilin cDNA kindly provided by C. Gasser (University of California, Davis) (Gasser et al., 1990).

### In Situ Hybridization

Tomato root tips, 0.5 to 1.0 cm in length, were vacuum infiltrated with 4% paraformaldehyde in phosphate buffer, pH 7.4. Tissue was dehydrated in a successive ethanol and *t*-butanol graded series and embedded in paraffin medium (Paraplast, Sigma). Hybridizations were performed on 10- $\mu$ m-thick sections, deparaffinized, and rehydrated as described by Barker et al. (1988) and Cox et al. (1984).

To generate riboprobe templates, the inserts in plasmids pEB28 and pEB29 were amplified by PCR using the M13 forward and reverse primers. The PCR products were purified, and sense and antisense [<sup>35</sup>S]UTP-labeled riboprobes were synthesized by *in vitro* transcription using T7 RNA polymerase according to the manufacturer's instructions (Promega). Probes were purified and then sheared to an average size of 150 bp, and hybridizations were conducted overnight at 42°C according to the method of Cox et al. (1984). Unhybridized probes were removed by treatment with RNase A. The slides were washed extensively, coated with nuclear track emulsion (NTB-2, Kodak), and then exposed for 2 weeks. After development, slides were stained with 0.01% toluidine blue, dehydrated, and mounted in Permount (Fisher Scientific). Photomicrographs were taken on a Nikon microphot-SA microscope fitted with a dark-field or a bright-field condenser.

### Heterologous Protein Expression

The truncated LeMir fragment was released from the introduced *Eco*RI sites of pEB29 and subcloned into the *Eco*RI site of the yeast (*Pichia pastoris*) expression vector pHILS-1 (Invitrogen). The new plasmid was linearized at the unique *Bgl*III site in the vector and transformed into *P. pastoris* strain GS115 by the combined LiOAC/electroporation method (Ausubel et al., 1987). Positive transformants were selected, and the transformed line EBY5 was chosen for subsequent work.

### Protein Purification and Antibody Preparation

A 100-mL culture of *P. pastoris* line EBY5 was induced according to the supplier's instructions (Invitrogen). Cells were pelleted at 2,500g for 15 min at 4°C, and the supernatant was collected and spun at 28,000g and 4°C to remove particles. The supernatant was concentrated to 10 mL in a Centriprep-10 column (Amicon, Beverly, MA). The concentrate was spin-dialyzed against binding buffer (10 mM NaCl, 25 mM Bis-Tris, pH 6.0, and 0.1 mM EDTA). Concentrated protein was loaded onto a DEAE-Sephadex A-25 column equilibrated with binding buffer, and eluted with a concentration gradient of NaCl increasing by 50 mM steps in 25 mM Bis-Tris, pH 6.0, 0.1 mM EDTA. Eluted LeMir was detected from fractions with 100 mM or greater NaCl as single bands after SDS-PAGE and staining with Coomassie blue. Fractions between 100 and 200 mM NaCl were pooled, concentrated to 10 mL, run on SDS-PAGE, and electroeluted from the gel. Eluted protein was dialyzed against 1× PBS, and 330  $\mu$ L was injected into a New

Zealand White rabbit three times at 2-week intervals to produce antibodies.

### Western-Blot Analysis

Tomato roots infected with an average of 40 nematodes per tip was harvested 12 h after inoculation, and 0.5 cm of root tips was frozen in liquid nitrogen. Total SDS-soluble proteins were extracted by grinding 75 frozen root tips in 100  $\mu$ L of extraction buffer (250 mM Tris, pH 6.8, 0.2% 2-mercaptoethanol, and 0.2% SDS). After microcentrifugation for 5 min, the protein concentration in the supernatant was determined by Bradford analysis (Bio-Rad). Ten micrograms of total protein was fractionated by SDS-PAGE and electroblotted onto nitrocellulose. Protein detection was performed using an enhanced chemiluminescence western-blotting system (Amersham). Membranes were prehybridized in TBS-T (20 mM Tris, pH 8.0, 137 mM NaCl, 0.2% Tween) plus 5% nonfat dry milk and washed in TBS-T. Incubation with the anti-LeMir immune serum was at 1:10,000 dilution in TBS-T. A secondary hybridization with donkey anti-rabbit antibody linked to horseradish peroxidase was at a dilution of 1:5,000. Signal was detected according to the instructions of the manufacturer (Amersham).

Tomato callus tissue derived from an F<sub>1</sub> hybrid line, ACE × Early Pac, was kindly donated by Bradford Hall (Center for Engineering Plants for Resistance Against Pathogens, Davis, CA).

### Seedling Squash-Immunoprint Analysis

cv VFN8 tomato seeds were surface sterilized, germinated on moistened filter paper in the dark for 5 d, and then moved into the light for another 3 d. Immunoprint analysis was adapted from the method of Terras et al. (1993). Seedlings with roots about 6 cm long were gently laid between two layers of nitrocellulose that had been moistened with TBS (20 mM Tris, pH 8.0, 137 mM NaCl). This assembly was then placed between two rectangles of blotting paper, which were sandwiched between two glass plates. Pressure was applied to the glass plates to force the release of the cellular contents and the deposition of soluble plant proteins onto the nitrocellulose. Protein deposition was detected by staining with 0.1% (w/v) amido black (naphthol blue-black) in 25% isopropanol and 10% acetic acid for 1 min and then destaining with several washes in 25% isopropanol and 10% acetic acid. LeMir protein was detected as described for western-blot analysis. As a positive control, mouse antiserum to tomato Rubisco (a generous gift of Susan Jenkins at the laboratory of William Gruissem, University of California, Berkeley) was used at a dilution of 1:5000 in TBS-T.

### Wounding Assays

Plants were grown in sand at 25°C, 75% RH, and a 16-h photoperiod at 350  $\mu$ E m<sup>-2</sup> s<sup>-1</sup> PAR. After 6 weeks, tissue was wounded according to the method of Lincoln et al. (1993). Root tissue and leaves were harvested and cut into

1-cm segments or squares. Tissue was crushed with a pair of forceps and left to incubate on filter paper moistened with 50 mM potassium phosphate buffer, pH 7.0, containing 50 µg/µL chloramphenicol, and were then frozen in liquid nitrogen at the indicated times.

**Analysis of Root Exudates**

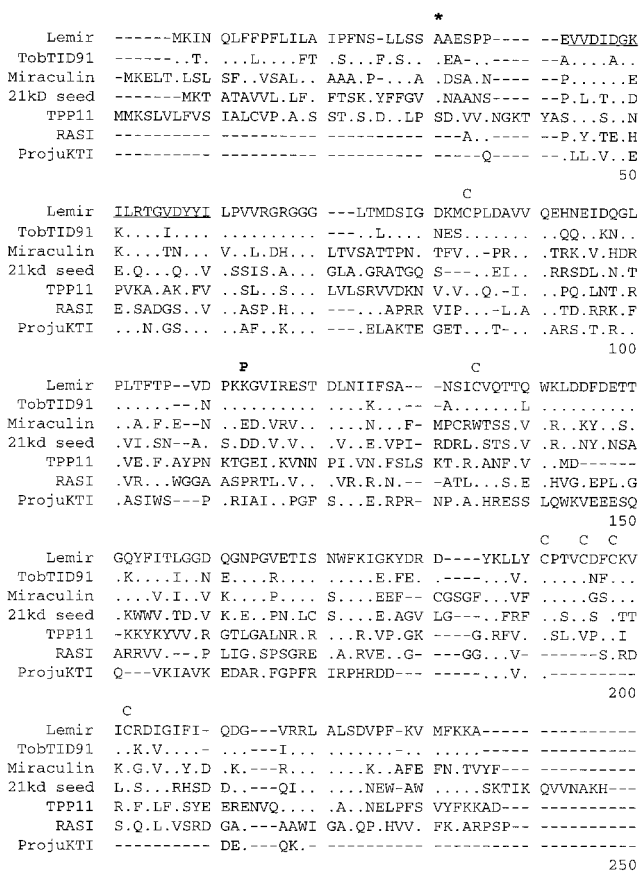
A rectangle of blotting paper was placed into the inside cover of a plastic tray and inserted into a Sunbag (44 cm × 20.5 cm with a 24-mm, 0.02-µm filter disc, Sigma) and autoclaved. Sterile 10 mM Mes, pH 6.0, was applied to saturate the filter paper in the trays, and a layer of ultra-clear cellophane (Research Products International, Mt. Prospect, IL) was placed over the filter paper. Cellophane sheets were prepared by autoclaving in two changes of 2% NaHCO<sub>3</sub>, 1 mM EDTA, followed by washing and autoclaving twice in deionized water. Tomato seeds (0.35 g) were surface sterilized and laid onto the membrane in a row along the length of the tray. The trays were resealed in the Sunbags and placed at an approximately 60° angle. Seeds were allowed to germinate in the dark, and then aligned seedlings were removed and rolled together into a cylinder shape.

The roots were immersed in 15 mL of 1 mM Mes, pH 6.0, in a 50-mL beaker and shaken gently for 1 h. Root exudates were passed through a 400-mesh screen (Bellco, Vineland, NJ) and a 0.45-µm filter (Millipore), and were then lyophilized and resuspended in 100 µL of extraction buffer before western-blot analysis. Immune serum to carrot EF-1α, a cytoplasmic marker (a generous gift of Richard Cyr, Pennsylvania State University, University Park), was used as a control at a dilution of 1:20,000.

**RESULTS**

**Isolation of Full-Length *LeMir* cDNA and Genomic DNA**

Northern-blot analysis indicated that the *LeMir* transcript was approximately 800 nucleotides, whereas the 23a cDNA was only 540 bp (Lambert, 1995). A modified RACE-PCR method was used to obtain the 5' end of the cDNA. Southern-blot analysis carried out under high stringency with clone 23a indicated that *LeMir* is a single-copy gene (Lambert, 1995). Using 23a cDNA as a probe, a clone of *LeMir* was identified from a tomato genomic library. The sequences of the full-length composite cDNA and the corresponding genomic region were determined. Comparison of the cDNA to the genomic DNA sequence revealed that *LeMir* contains no introns. Where differences were found between the cDNA and genomic DNA sequence, the genomic DNA sequence was substituted because the cDNA was obtained from a library generated using sequences amplified by *Taq* polymerase, which can introduce errors. The composite cDNA is 791 bp and has an open reading frame coding for a polypeptide of 205 amino acids (22.8 kD) (Fig. 1). There is a 46-nucleotide 5'-untranslated sequence before the translation start and a 127-nucleotide 3'-untranslated region with a polyadenylation signal starting at nucleotide 774.



**Figure 1.** Deduced 205-amino acid sequence of *LeMir* and alignment with similar sequences. Residues identical to *LeMir* (dots) and gaps introduced for the alignment (dashes) are indicated. The asterisk marks the predicted cleavage site for the putative signal sequence. The N-terminal signature sequence of the soybean trypsin-inhibitor family (L/I/V/M)-X-D-X-(E/D/N/T/Y)-(D/G)-(R/K/H/D/E/N/Q)-X-(L/I/V/M)-X(5)-Y-X-(L/I/V/M) is underlined (Genetics Computer Group motif program; Laskowski and Kato, 1980). "P" indicates a potential proteinase-inhibitor reactive site. The program PILEUP (Genetics Computer Group) was used with a gap-length weight of 0.1. The conserved Cys residues are indicated by a "C" above the *LeMir* sequence. Sequences aligned with *LeMir* are: TobTID91, the deduced protein sequence of a gene expressed in tobacco tumors (Fujita et al., 1994; Karrer et al., 1998); Miraculin, a taste-modifying protein from *R. dulcifica* (Theerasilp et al., 1989); 21-kD seed, a major seed protein from cacao (Spencer and Hodge, 1991); TPP11, the coding sequence of a gene expressed in tomato floral tissue (Milligan and Gasser, 1995); RASI, a rice α-amylase/subtilisin inhibitor (Ohtsubo and Richardson, 1992); and ProjuKTI, a trypsin inhibitor from the seeds of *Prosopis juliflora* (Ngreiros et al., 1991).

The first 25 amino acids at the N terminus have the properties of a typical signal peptide in which a positively charged amino acid (Lys-2) is followed by a hydrophobic core of amino acids and a putative cleavage site at residue 24 or 25 (Von Heijne, 1988). Cleavage at this site would produce a mature protein of about 20 kD.

***LeMir* Expression Patterns**

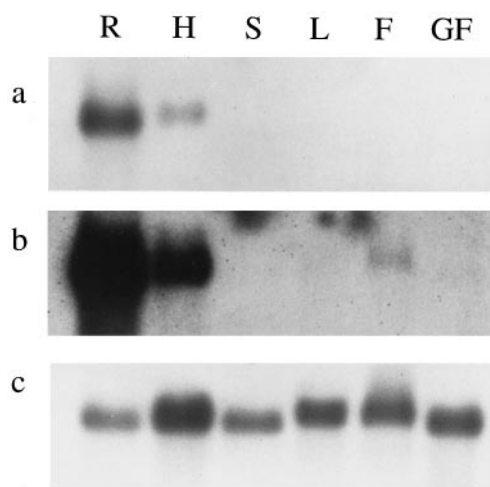
To determine the pattern of *LeMir* expression, a northern blot of RNA extracted from root, stem, leaf, flower, fruit,

and hypocotyl was hybridized with *LeMir* cDNA. A band of the expected size (800 nucleotides) was detected in the lanes containing root, hypocotyl, and flower RNA (Fig. 2). The signal intensity was the strongest with the root RNA; a band was visible only after long exposure in flower RNA, and no signal was detected in leaf, stem, or fruit RNA.

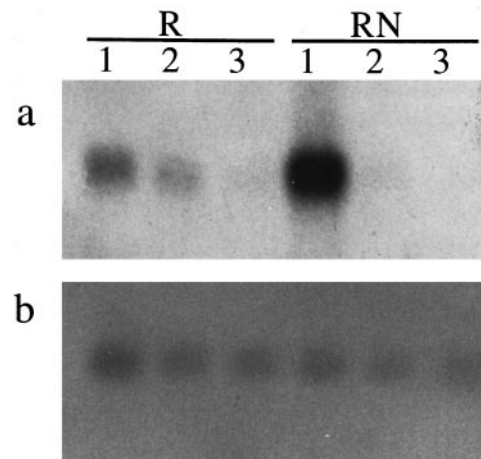
The spatial pattern of *LeMir* induction by nematode infection in tomato seedling root tips was examined by extracting RNA from three 0.5-cm sections cut sequentially from the terminal 1.5 cm of root tips of infected and uninfected tomato seedlings. Northern blots with RNA from these root sections showed that *LeMir* was expressed more highly in the terminal 0.5 cm in uninfected root tips than in the next centimeter (Fig. 3). In nematode-infected root tips, *LeMir* expression increased to about 6.5-fold that in uninfected root tips. There was no increase, and perhaps even a decrease, in transcript levels in adjacent sections of the root. Because nematodes predominantly infect the terminal 0.5 cm of the root tip, these results indicate that at 11 h after infection, *LeMir* induction is localized at or near the site of infection.

#### Localization of *LeMir* Expression within Root Tips

In situ hybridization was carried out to localize the expression of *LeMir* mRNA within root tips and to determine where the expression pattern changes after nematode infection. Root tips were infected with 25 nematodes per root for 24 h. The infected roots contained, on average, 15 nematodes per tip. This infection time and nematode number resulted in roots with individual nematodes distributed throughout the root-tip region and migrating to the zone of differentiation, where giant cell initiation occurs in susceptible roots. This distribution of nematodes allowed the visualization of gene expression in relation to individual



**Figure 2.** Tissue specificity of *LeMir* expression. Total RNA was obtained from cv VFNT cherry tomato mature root (R), stem (S), leaf (L), flower (F), and green fruit (GF); hypocotyl RNA (H) was from VFNT seedlings. RNA was fractionated on a 2% agarose gel and blotted onto a nylon membrane. The blot was hybridized to labeled *LeMir* cDNA probe 23a and exposed for 1 d (a) or 8 d (b). The blot was stripped and then hybridized to cyclophilin cDNA (c).



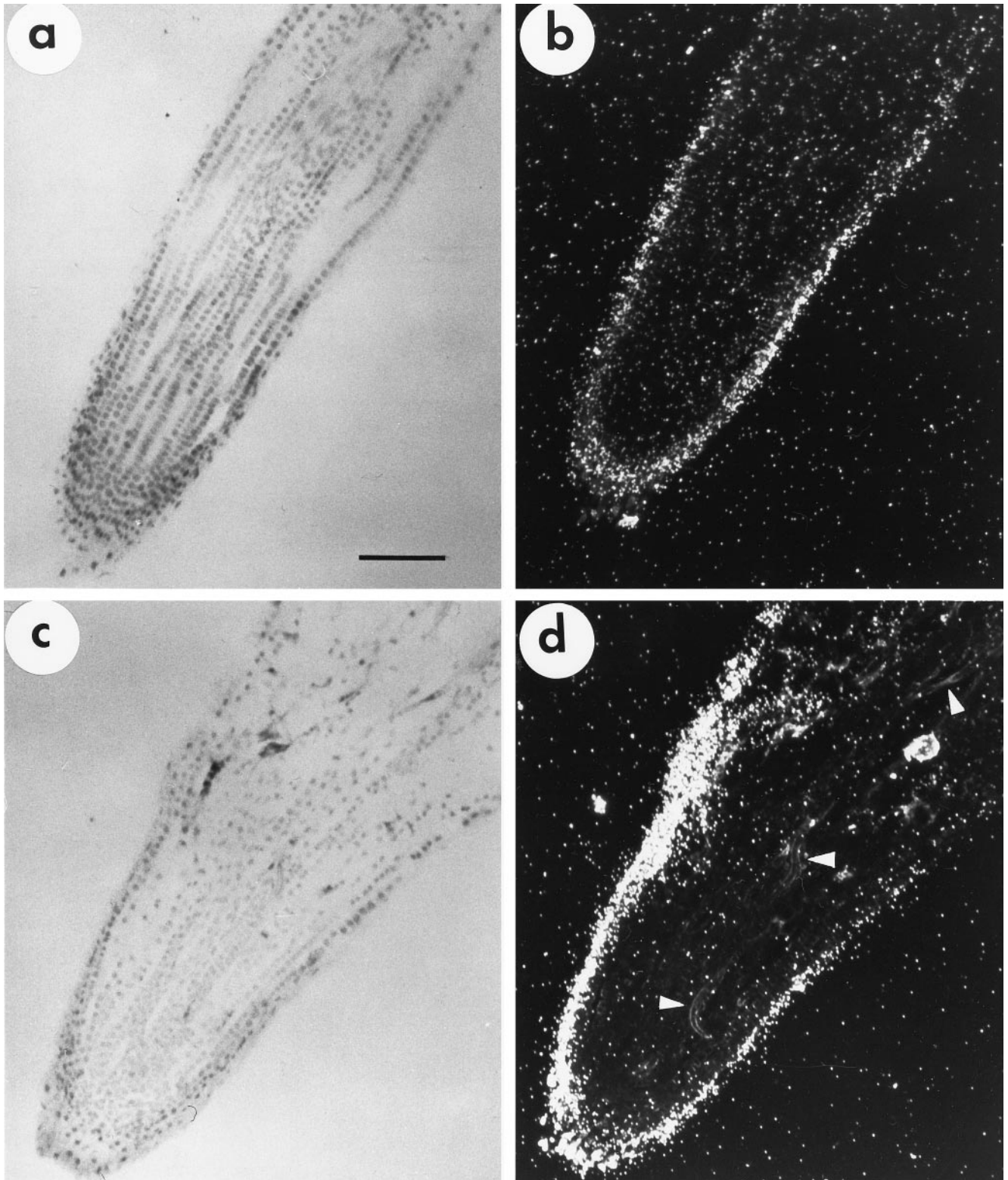
**Figure 3.** Spatial expression of *LeMir* in nematode-infected and uninfected tomato root. a, Northern blot of total RNA from root sections hybridized with labeled *LeMir* cDNA. Lanes 1, 2, and 3 contain RNA from sequential 0.5-cm sections of tomato seedling roots, with lane 1 containing the RNA from the tip. Corresponding regions were excised from both infested (RN) and uninfested (R) plants. cv VFNT seedlings were infested with second-stage juveniles for 11 h. Roots contained an average of 60 nematodes per root tip at the time of RNA extraction. b, The blot was stripped and then re-probed with tomato cyclophilin.

nematodes in several phases of migration to their feeding site.

Sectioned root tips infected with nematode juveniles and uninfected controls were hybridized to sense and antisense *LeMir* riboprobes. In uninfected tomato, strong expression was seen in the root cap and epidermis of the root tip with the antisense probe (Fig. 4, a and b). The expression of *LeMir* decreased with distance from the root tip. Nematode-infected root sections showed a similar overall pattern of *LeMir* expression. However, a sharp increase in expression of *LeMir* occurred in the epidermal and cortical cells, possibly as a result of nematode penetration (Fig. 4d). No dramatic increase in expression was observed in cells in contact with the nematodes. With the sense riboprobe, no grains above background were observed in uninfected or infected roots (data not shown).

#### Characterization and Localization of *LeMir* Gene Product

The yeast *P. pastoris* was used to generate the *LeMir* gene product (LeMir). A chimeric construct was created that replaced the putative *LeMir* signal sequence with that of *P. pastoris* acid phosphatase. Once integrated in the *P. pastoris* genome, expression of recombinant protein is controlled by an alcohol oxidase promoter, which is activated by the addition of methanol to the medium. After induction, a predominant band of 25 kD was detected in the medium. This protein was purified and concentrated, as described in "Materials and Methods," and was then used to generate anti-*LeMir* immune serum. The antiserum detected a single band of 24 kD in total protein extracts from tomato root tips (Fig. 5A). Hybridization with preimmune serum did not detect this band (data not shown). The intensity of the band



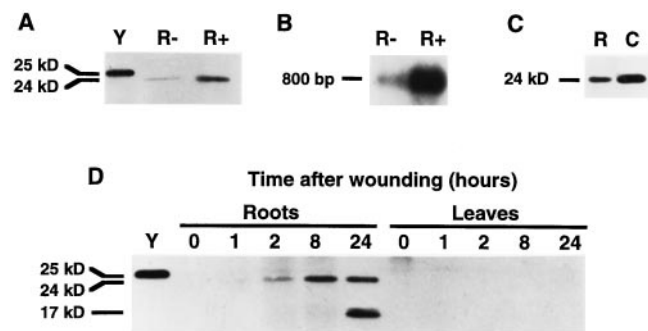
**Figure 4.** In situ localization of *LeMir* transcript in tomato root tips. Bright-field (a and c) and dark-field (b and d) micrographs of root tips hybridized with *LeMir* antisense probe. The hybridization signal is visible as white spots in the dark-field micrographs. Uninfected (a and b) and infected (c and d) root tips are compared. In d, several nematodes can be seen migrating through the cortex (indicated by white arrowheads). Black bar in a represents 100  $\mu\text{m}$ .

increased strongly in root tips after nematode infection (Fig. 5A). Northern-blot analysis performed on tissue from the same experiment revealed that *LeMir* mRNA levels were also induced (Fig. 5B). This confirmed previous results that transcript levels were induced after nematode infection. Because TID91, a gene with 81% identity to *LeMir*, is strongly expressed in tobacco callus tissue (Fujita et al., 1994), we tested tomato callus tissue for the presence of LeMir. Western-blot analysis revealed that LeMir is expressed in this tissue (Fig. 5C).

In situ analysis showed strong induction of *LeMir* mRNA expression in apparently damaged tissues that may have been breached by the nematode. This suggested that wounding might induce LeMir protein levels. To determine if this was the case, leaf and root tissue from 6-week-old tomato plants were cut into small pieces, wounded, and incubated in phosphate buffer. A detectable increase of LeMir in roots was observed within 1 to 2 h of wounding (Fig. 5D). A second band of 17 kD was detected at 24 h after incubation. In the leaf, no induction was observed in the experiment shown in Figure 5D; however, very weak induction of LeMir was occasionally observed in leaves in some experiments, including the presence of the 17-kD band (data not shown).

#### LeMir Localization in Seedlings

A profile of LeMir localization in the young seedling was obtained by producing "immunoprint squashes" on nitrocellulose. LeMir was detected throughout the root (Fig. 6, B and C). Expression appeared highest at the root/shoot



**Figure 5.** Western-blot analysis of LeMir expression in tomato tissue after nematode infection or wounding. **A**, Analysis of LeMir after infection of tomato root tips with nematodes. Total SDS-soluble proteins were extracted from root tissue 12 h after inoculation. Ten micrograms of protein was analyzed per lane. Blots were probed with anti-LeMir immune serum. Lane Y, 0.04 ng of purified LeMir from the yeast *P. pastoris* was run as a positive control; lane R-, uninfected root tips; lane R+, root tips from seedlings inoculated with *M. javanica*. **B**, RNA blot probed with *LeMir*. RNA was extracted from a subsample of root tips from the same experiment shown in **A**. Equal amounts of RNA were loaded on each lane. **C**, Western-blot detection of LeMir in tomato callus. Lane R, Ten micrograms of protein extracted from an uninfected seedling root; lane C, 10  $\mu$ g of protein from tomato callus. **D**, Induction of LeMir by wounding. Tissue samples were collected at the indicated times after wounding and frozen in liquid nitrogen. Protein was extracted and LeMir was detected by western-blot analysis.

junction, diminished progressively toward the zone of elongation, and then increased again toward the root apex. This expression pattern is consistent with northern-blot and in situ localization patterns seen in the root tip, where mRNA levels in the terminal 1.5 cm were examined (Figs. 3 and 4d). There is a sharp loss of expression in the hypocotyl directly above the root/shoot junction. Little expression is observed in the shoot except for a localized signal at the tips of the cotyledons. As a positive control, an antibody raised against Rubisco shows localization in the shoot (Fig. 6D).

#### Detection of LeMir in Root Exudates

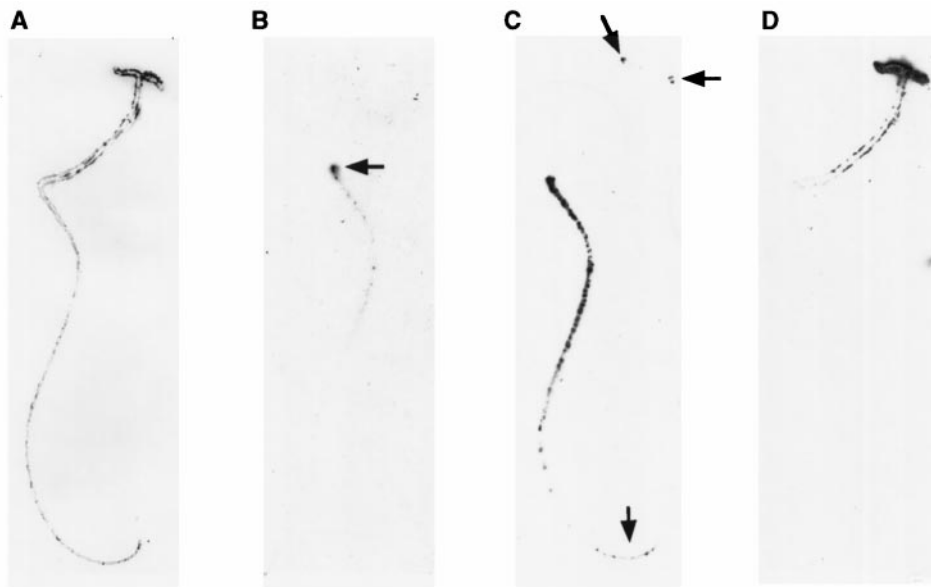
The predicted amino acid sequence of LeMir contains a putative signal sequence indicative of targeting into the ER and possible secretion out of the cell. Also, *LeMir* mRNA was found by in situ analysis to be mainly present in cells found at the periphery of the root tip. These observations suggested that LeMir might be secreted out to the surrounding environment from the root cap and epidermis. To test this possibility, roots of 6-d-old seedlings were immersed in buffer for 1 h, and products exuded from the root were collected. Exudates were found by western-blot analysis to contain the same 24-kD band detected in root tips (Fig. 7A). If LeMir was released into the medium by a cell-lytic process, one would expect to find intracellular proteins in these exudates. To test for the presence of a cytoplasmic protein, exudates were probed with an interspecific, cross-reactive antibody raised against carrot EF-1 $\alpha$ . This marker represents 5% to 10% of the total soluble cytoplasmic protein in plant roots (Durso et al., 1996). This antibody recognizes a 50-kD peptide in root tissue extracts; however, no band was present in root exudates (Fig. 7B). These results indicate that LeMir is secreted out of the root.

## DISCUSSION

#### Sequence Similarity with Known Proteins

A database similarity search revealed that the deduced amino acid sequence of LeMir has similarity to several cloned plant genes of unknown function (Fig. 1). The highest similarity, 81% identity at the amino acid level, is to a deduced protein from a cDNA, TID91, representing a gene expressed in stress-induced, genetic tumor tissues found in interspecific hybrids between *Nicotiana glauca* and *Nicotiana langsdorffii*. TID91 was demonstrated to be strongly expressed in tobacco callus tissue but absent in leaves and stems (Fujita et al., 1994). Expression in roots was not examined. Other related sequences of unknown function include a 21-kD seed protein (42% identity) from cacao (Spencer and Hodge, 1991), and the deduced protein corresponding to a tomato cDNA clone, TPP1 (36% identity), which is highly specific to floral tissues (Milligan and Gasser, 1995). Finally, LeMir shows 38% identity at the N terminus to G2, a protein of unknown function found in developing taro corms (de Castro et al., 1992).

The highest similarity of LeMir to a protein with known activity is to miraculin (54% identity; Fig. 1) (Masuda et al.,



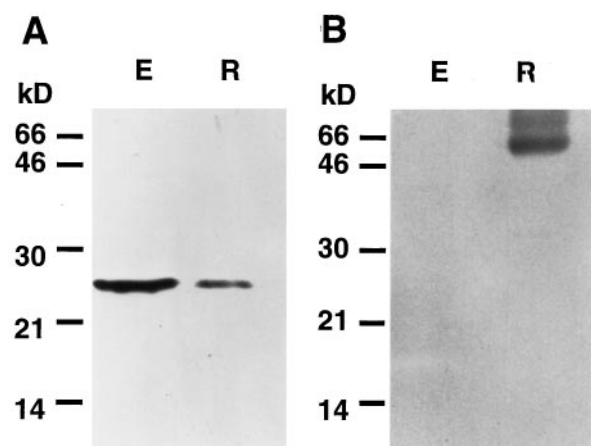
**Figure 6.** Root squash-immunoprint analysis of LeMir localization. A through D portray the same 8-d-old seedling stained for total protein with amido black (A) and probed with LeMir antiserum (B and C; C is a longer exposure of B). In B, an arrow points to the site of heaviest LeMir expression in the root at the root/shoot junction. In C, arrows point to heightened expression at the tips of the cotyledons and near the root tip. D, Immunoprint probed with antiserum to the small subunit of Rubisco from tomato.

1995). Miraculin modifies taste perception but has no taste itself (Theerasilp and Kurihara, 1988). LeMir and miraculin share six Cys residues in approximately the same positions (Fig. 1). In miraculin, these residues have been shown to be involved in intrachain disulfide linkages (Igeta et al., 1991). Miraculin has an additional Cys that forms an interchain disulfide bridge. This Cys is not present in LeMir, suggesting that LeMir may not have the tertiary structure of miraculin. Although it is highly similar to miraculin in size, LeMir is an acidic protein (predicted pI 4.58 for the mature protein), and the deduced sequence contains no glycosylation motifs, whereas miraculin is a basic glycoprotein. Therefore, the properties and possibly the functions of LeMir and miraculin may be quite different.

Sequence analysis indicates that LeMir and miraculin belong to the soybean trypsin-inhibitor family. Members of this family are characterized by the presence of an N-terminal signature sequence (Fig. 1). Many but not all members have been shown to have inhibitory activity against a range of Ser proteinases (Laskowski and Kato, 1980). The highest similarity (30% amino acid identity) of LeMir to a known enzymatic inhibitor is to a bifunctional  $\alpha$ -amylase/subtilisin inhibitor of rice, RASI (Ohtsubo and Richardson, 1992).  $\alpha$ -Amylase/subtilisin inhibitors are thought to function as defense proteins against insects, fungi, bacteria, and viruses (Lazaro et al., 1988). RASI has been shown to inhibit subtilisin, a microbial proteinase, and an  $\alpha$ -amylase from the larvae of the red flour beetle (Ohtsubo and Richardson, 1992).

Kunitz trypsin inhibitors function by binding tightly in a 1:1 ratio to the active site of Ser proteinases. Proteinase binding occurs at a characteristic peptide loop of the inhibitor that is a substrate for the proteinase (Kojima et al.,

1993). An amino acid residue (either Lys or Arg) in the binding loop, denoted P1, is responsible for the specificity of trypsin (Laskowski and Kato, 1980). LeMir has a Lys residue at a position that aligns with the P1 site of known trypsin inhibitors, such as the major trypsin inhibitor from seeds of *P. juliflora* (Fig. 1) (Negreiros et al., 1991). However, our preliminary tests using *P. pastoris*-expressed LeMir have not detected proteinase-inhibitory activity against trypsin or chymotrypsin (data not shown).



**Figure 7.** Detection of LeMir in exudates of tomato seedling roots. Roots of 6-d-old tomato seedlings were immersed in buffer and incubated for 1 h. Root exudates were collected as described in "Materials and Methods." Total SDS-soluble proteins were extracted from the same roots. Ten microliters of exudate (E) and 13  $\mu$ g of root extract (R) was separated by SDS-PAGE and transferred to nitrocellulose to detect the presence of LeMir (A) or carrot EF-1 $\alpha$ , a 50-kD cytoplasmic protein (B).



### Expression Pattern and Possible Role of *LeMir*

A clone corresponding to TID91, the tobacco gene with high similarity to *LeMir*, was identified in a cDNA library from tobacco leaves undergoing a hypersensitive response (Karrer et al., 1998). A tobacco mosaic virus vector expressing this protein triggered a localized necrosis or hypersensitive response on susceptible tobacco, similar to that triggered by the wild-type virus on resistant plants. Karrer et al. (1998) suggest that this protein may act as a positive regulator of the hypersensitive response. If this were true for *LeMir* in tomato, we would expect the levels of induction by nematode infection to be higher in plants with the resistance gene *Mi*, in which nematode infection triggers a hypersensitive response, than in susceptible tomato. In some experiments we have found that induction in root tips is greater in plants with the *Mi* gene than in susceptible tomato (Williamson et al., 1994). However, in other experiments, we have found equal induction in resistant and susceptible seedlings after nematode infection (B. Ferrie and V. Williamson, unpublished data).

We have not been able to determine whether the differences between experiments are the result of experimental variables or if there is a difference in the timing of induction between resistant and susceptible plants. The hypersensitive response mediated by *Mi* occurs in the cells of the meristem and the developing vascular cylinder, which does not correspond to the cellular location of *LeMir* induction after nematode infection (Fig. 4) and thus is not consistent with a role for this protein as a positive regulator of the hypersensitive response. Nevertheless, additional time points should be examined before this possibility is ruled out.

Analysis of *LeMir* expression patterns reveals that this gene is expressed mainly in the root. The highest levels of protein occur at the root/shoot junction, a common penetration and colonization site for a variety of plant pathogenic organisms such as *Agrobacterium tumefaciens*, the causative agent of the crown gall disease. Weak expression of *LeMir* message was also observed in flowers. Similarly, the expression of pathogenesis-related proteins is commonly found in flowers (Lotan et al., 1989).  $\alpha$ -Amylase/subtilisin inhibitors, which show sequence similarity to *LeMir*, are frequently expressed in flowers (Mundy et al., 1984). *LeMir* is present in the tips of the cotyledons of germinated seedlings (Fig. 6). Transgenic plants carrying a *LeMir* promoter::reporter fusion gene would be useful in determining if other organs and tissues in the shoot express this gene.

Plant roots are exposed to a wide variety of pathogens, including plant-parasitic nematodes. *LeMir* expression is induced rapidly and locally at the site of nematode infection in tomato. This response may be partly attributable to wounding or to elicitors generated from or by the nematode. Hansen et al. (1996) examined the expression of the wound-inducible *wun1* promoter fused to the reporter gene *GUS*, and found that expression could be detected rapidly and transiently after infection by cyst nematodes in potato roots. Root-knot nematode juveniles cause considerably less cell damage than cyst nematodes, but also induce

defense genes (Williamson and Hussey, 1996). However, only a modest and much slower induction of *wun1* was observed after root-knot nematode infection. In contrast, we have shown that *LeMir* is induced by the root-knot nematode as well as by wounding in the roots. Whether *LeMir* is induced solely by wound damage from the nematode or from other aspects of nematode infection as well remains to be determined.

When a nematode infects a root tip, it provides an opportunity for soil-borne pathogens to enter through the breach in the epidermis it creates. Up-regulation of *LeMir* in damaged root tissue may function to prevent pathogen ingress into the damaged area, consequently stopping systemic plant infection. Many pathogen- or pest-induced genes have been isolated and characterized. Several of these possess antipathogen activity (Bowles, 1990; Ryan, 1990; Lamb et al., 1992). However, genes induced by one pathogen may protect against an entirely different pathogen (Van Loon and Van Kammen, 1970; Rigden and Coutts, 1988). For example, thaumatin-like proteins are induced by a variety of biotic and abiotic stresses but have antifungal activity (Vigers et al., 1991). Examples of thaumatin-like proteins include PR-5, which is up-regulated as part of the N gene response to the tobacco mosaic virus, and osmotin, which is induced by salt stress. Thaumatin-like proteins have many similarities to *LeMir*: both show homology to proteins that have the ability to affect taste in higher primates, both have homology to protease inhibitor families, and both have a high number of conserved Cys residues. Thaumatin-like proteins act by permeabilizing the fungal plasma membrane (Roberts and Selitrennikoff, 1990; Hejgaard et al., 1991). Whether *LeMir* has antifungal capabilities or other antibiotic properties remains to be determined.

The protein encoded by *LeMir* contains a putative signal peptide, indicating that it enters the secretory pathway. We have shown that *LeMir* is found in tomato seedling exudates. Several proteins, including acid phosphatase from lupin (Tadano et al., 1993) and catalase from maize roots (Salguero and Bottger, 1995), have been observed to be released from the roots of plants into the external environment. In *Trichosanthes kirilowii* root cultures, defense-related proteins such as chitinases have been detected in root exudates (Savary and Flores, 1994; Savary et al., 1997). To our knowledge, *LeMir* is the only member of the protease-inhibitor family shown to be naturally deposited outside of the roots. However, in tomato plants genetically engineered to ectopically express the root-proteinase inhibitors I and II, which are normally localized in the leaves, these proteins can be found in root washes (Narvaez-Vasquez et al., 1993).

Our results show that the deposition of *LeMir* outside of the root occurs through a secretory rather than a cell-lytic process. Proteins secreted from the root cap have been shown to significantly affect microbial populations in the rhizosphere (Hawes, 1990). Cells released from the root cap into the rhizosphere, otherwise known as "root border cells," secrete up to 25% of the protein they produce directly into the soil (Brigham et al., 1995). *LeMir* mRNA expression is highest in the root cap and in the cell layers

residing on the periphery of the root tip. These cell layers are a logical site to produce protein that is secreted into the rhizosphere. Whether *LeMir* is expressed in tomato root border cells remains to be determined.

Our characterization of *LeMir* raises many questions. Although our results suggest that *LeMir* may be involved in defense, we cannot discount the possibility that it has other roles in plant maintenance, development, or signaling with the environment. For example, an alternative potential role for *LeMir* is interaction with beneficial soil microorganisms in a symbiotic manner. Root exudates have been shown to affect the microflora in the rhizosphere surrounding the roots (Gochnauer et al., 1990; Hawes, 1990). Plant roots often rely on symbiotic relationships with microorganisms in the rhizosphere for defensive assistance (Handelsman and Stabb, 1996). *LeMir* could thus have an advantageous effect on beneficial microorganisms in the rhizosphere. However, the expression pattern of *LeMir*, its up-regulation by nematodes and wounding, and its membership in the soybean trypsin-inhibitor family are consistent with the hypothesis that this protein is involved in plant defense. Clearly, more work is required to define the role of *LeMir* in tomato roots and in the rhizosphere.

#### ACKNOWLEDGMENT

We thank Bonnie Ferrie for helpful comments on the manuscript.

Received January 16, 1998; accepted June 19, 1998.

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The accession number for the sequence reported in this article is U70076.

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