

Early Root Response to *Meloidogyne incognita* in Resistant and Susceptible Alfalfa Cultivars¹

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Abstract: The early events of *Meloidogyne incognita* behavior and associated host responses following root penetration were studied in resistant (cv. Moapa 69) and susceptible (cv. Lahontan) alfalfa. Ten-day-old seedlings of alfalfa cultivars were inoculated with second-stage juveniles (J2) and harvested 12, 24, 48, and 72 hours and 7, 14, and 21 days later. Both cultivars supported similar root penetration and initial J2 migration. By 72 hours after inoculation the majority of J2 were amassed inside the vascular cylinder in roots of susceptible Lahontan, while J2 had not entered the vascular cylinder of resistant Moapa 69 and remained clumped at the root apex. Nematode development progressed normally in Lahontan, but J2 were not observed in Moapa 69 after day 7. The greatest differences between RNA translation products isolated from inoculated and uninoculated roots of Lahontan occurred 72 hours after inoculation. Only minor differences in gene expression were observed between inoculated and uninoculated Moapa 69 roots at 72 hours. Comparison of translation products from inoculated versus mechanically wounded Lahontan roots revealed products that were specific to or enhanced in nematode-infected plants. Moapa 69 appears to possess a type of resistance to *M. incognita* that does not depend on a conventional hypersensitive response.

Key words: alfalfa, gene expression, host response, Lahontan, *Medicago sativa*, *Meloidogyne incognita*, Moapa 69, resistance, RNA, root-knot nematode.

Root-knot nematodes (*Meloidogyne* spp.) are widely distributed and economically important sedentary endoparasites of agriculturally important plants. During the first few days following root penetration, second-stage juveniles (J2) initiate multinucleate giant cells (11,24,39,42), which function thereafter as nutrient sinks for the developing nematode (28). Unique behavior is exhibited by the J2 before giant cell induction. The nematode follows a circuitous intercellular journey among cortical cells from the point of root penetration in the zone of elongation to the root apex, where the direction of migration is reversed and the J2 moves upward into the developing vascular cylinder (42). Giant cell induction results from repeated stylet probing accompanied by delivery of esophageal gland secretions to host cells in the developing stele (8).

Plants are characterized as resistant or susceptible to sedentary endoparasites based on corresponding nematode reproduction (7). Resistance mechanisms and other factors influencing host response have been reviewed elsewhere (7,22,37). The most common postinfectious resistance to *Meloidogyne* spp. involves hypersensitivity, in which necrosis occurs in giant cells or plant cells adjacent to these feeding sites (21,22). Hypersensitive responses have been associated with resistance to *M. incognita* in cultivars of snap bean (13), soybean (24,39), tobacco (36), and tomato (11,32), while both the presence and absence of cell necrosis have been reported in cotton (4,29). Resistance to *M. incognita* in alfalfa, however, occurs in the absence of any hypersensitive response (34). Reduction in the number of J2 inhabiting resistant alfalfa roots several days after infection has been reported for both *M. incognita* and *M. hapla* affecting nondormant and dormant cultivars, respectively (15,34).

The mechanisms involved in the susceptibility and resistance of host plants to nematodes have been the subject of speculation (35,37). While most of the molecular investigations have focused on mapping and chromosome walking toward the

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resistance genes (14,19,20), some efforts have been made to isolate and characterize cDNAs that represent genes that are specifically induced in the susceptible or resistant host (2,41). Wilson et al. (41) used a subtractive approach to construct a cDNA library of transcripts exhibiting up-regulated expression in giant cells of tomato. Lambert and Williamson (26) constructed PCR-based cDNA libraries to RNA isolated from infected root tips of nematode-resistant tomato followed by differential screening. Several cDNAs specific for the infected roots were isolated and characterized, but their relationship to resistance or susceptibility has not been determined. Conkling et al. (6) identified a root sequence (TobRB7) in tobacco that was highly expressed in giant cells and appears to be a putative water channel protein (30). Using a differential screening approach, Gurr et al. (16) identified a gene in potato that was expressed specifically in roots infected with *Globodera rostochiensis*.

Most molecular studies of plant-nematode interactions involving sedentary endoparasites have addressed responses in tomato, potato, and tobacco (2,6,17,26,30,40). We chose to study the interaction between alfalfa and *M. incognita* because the lack of a hypersensitive response in the nondormant resistant cultivar Moapa 69 (34) may indicate a mechanism of resistance to *M. incognita* that differs from those identified in tomato and tobacco. Similar resistance to *M. hapla* in dormant alfalfa cultivars also has been reported (15). Also, the high degree of characterization of molecular responses in alfalfa to other root pathogens (10,18) provides valuable information regarding root responses to invading organisms. The objective of this study was to identify nematode behavior and early molecular responses in roots of resistant and susceptible alfalfa infected with *M. incognita*.

MATERIALS AND METHODS

Nematode inocula: A population of *Meloidogyne incognita* (Kofoid & White)

Chitwood race 3 was isolated from cotton in New Mexico and maintained in the greenhouse on tomato, *Lycopersicon esculentum* Mill. cv. Rutgers. Inoculum was collected from nematode-infested tomato plants growing in aerated hydroponic culture to maximize recovery of large quantities of J2 (25). Freshly hatched J2 were collected twice daily from the hydroponic culture, quantified, and transferred to 10-day-old alfalfa seedlings.

Growth chamber experiments: Seeds of alfalfa (*Medicago sativa* L.) cultivars classified as resistant (cv. Moapa 69) and susceptible (cv. Lahontan) to *M. incognita* (3) were surface-sterilized in 0.5% sodium hypochlorite, and 100 seeds were planted in a 7.5 cm square by 10 cm tall Magenta box (modified Coplin jar) containing 100 cm³ of an autoclaved 2:1 mixture by volume of sand and Maricopa fine sandy loam. Texture of the mixture was a loamy sand (85% sand, 10% silt, 5% clay, pH of 7.7, 0.2% organic matter). Ten days after alfalfa seedling emergence (ca. 70 Lahontan or Moapa 69 seedlings per Magenta box), the soil in each Magenta box was infested with 2×10^4 *M. incognita* J2 suspended in 10 ml distilled water. Uninoculated controls received 10 ml distilled water. Nematode treatments were replicated eight times for each cultivar. Seedlings were grown in a growth chamber at 28 °C using cool white, very-high-output, fluorescent and incandescent lamps with a 16-hour photoperiod (photon flux density of 465 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Two inoculated and two uninoculated boxes of each cultivar were harvested 12, 24, 48, and 72 hours after inoculation. A similar study involved infesting soil in each box with 10^4 J2 and harvesting plants 7, 14, and 21 days after inoculation. Both experiments used a completely randomized design.

For the experiments described above, roots from one inoculated and one control box for each cultivar were washed free of soil, clipped below the stem, snap-frozen in liquid nitrogen, and stored at -80°C for future use. Roots from the remaining boxes were washed free of soil, clipped,

stained with acid fuchsin (5), and examined microscopically for the presence of *M. incognita*. Both studies were repeated with the same number of replications and conditions described above, except that staining was restricted to 10 randomly selected seedlings from each inoculated box to maximize the amount of frozen root tissue available for RNA extraction.

Since nematode entry into roots is accompanied by cell damage, it is likely that some of the gene products in the roots of the susceptible cultivar 72 hours after inoculation are induced in response to wounding. Host response to nonspecific wounding also was examined in a separate, completely randomized experiment using uninoculated seedlings of both alfalfa cultivars grown in the manner described above. Ten days after emergence, seedlings from four Magenta boxes of each cultivar were washed free of soil and roots were gently wounded with a fine-bristled wire brush to simulate nematode penetration and placed in water for 30 minutes before freezing. Roots from four additional boxes of each cultivar were clipped and snap-frozen, as previously described, except that the seedlings were not wounded. The 2D-gel profiles of the translation products of RNA from the wounded roots of the susceptible host were compared to those from the infected roots 72 hours after inoculation. The wound response was not examined in Moapa 69 because we were primarily interested in comparing gene products induced by mechanical wounding with those induced by nematode infection in susceptible plants.

Isolation and characterization of RNA: Total RNA was isolated from alfalfa roots using the LiCl precipitation procedure described by DeVries et al. (9) and quantified spectrophotometrically. Four micrograms of total RNA was translated in vitro using the wheat germ translation system (Promega Corp., Madison, WI) with ^{35}S -methionine (New England Nuclear, Boston, MA) as the tracer amino acid (1). The translation products were separated by two-dimensional sodium dodecyl sulfate

polyacrylamide gel electrophoresis (2D SDS-PAGE) and visualized using fluorography and autoradiography (27). The 2D SDS-PAGE procedure was similar to the protocol described by O'Farrell (31), except that 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) replaced Nonidet P-40 as the detergent in all isoelectric focusing (IEF) solutions (33) and IEF was run 14 hours at 400 volts, followed by 1 hour at 800 volts. The tube gel was then equilibrated in a solution containing 62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 5% β -mercaptoethanol, and 10% glycerol and mounted on a 12% SDS PAGE slab gel with a 30:0.8 ratio of acrylamide:bisacrylamide. Protein standards of known molecular weight were included in the SDS-PAGE.

RESULTS

Second-stage juvenile behavior in roots: Similar levels of infection and location of J2 within stained roots were observed between Lahontan and Moapa 69 cultivars 12 or 24 hours after inoculation (Fig. 1). Approximately two-thirds of all root tips were infected, and each root tip contained multiple J2. At 12 hours post inoculation, most J2 occupied the cortical tissue slightly above the root apex (Fig. 1A,E). After 24 hours, the majority of nematodes had migrated to the root tip in both cultivars (Fig. 1B,F). Different J2 behavior was observed between Lahontan and Moapa 69 cultivars 48 and 72 hours after inoculation (Fig. 1C,D,G,H). Nematodes were no longer clustered in root tips of susceptible Lahontan but had migrated upward into the developing vascular cylinder (Fig. 1C,D). After 72 hours, the majority of J2 were amassed inside the vascular cylinder and oriented parallel to the long axis of the root. In resistant Moapa 69, no J2 were observed entering the vascular cylinder (Fig. 1G,H) and J2 remained clumped and apparently disoriented at the root apex after 72 hours.

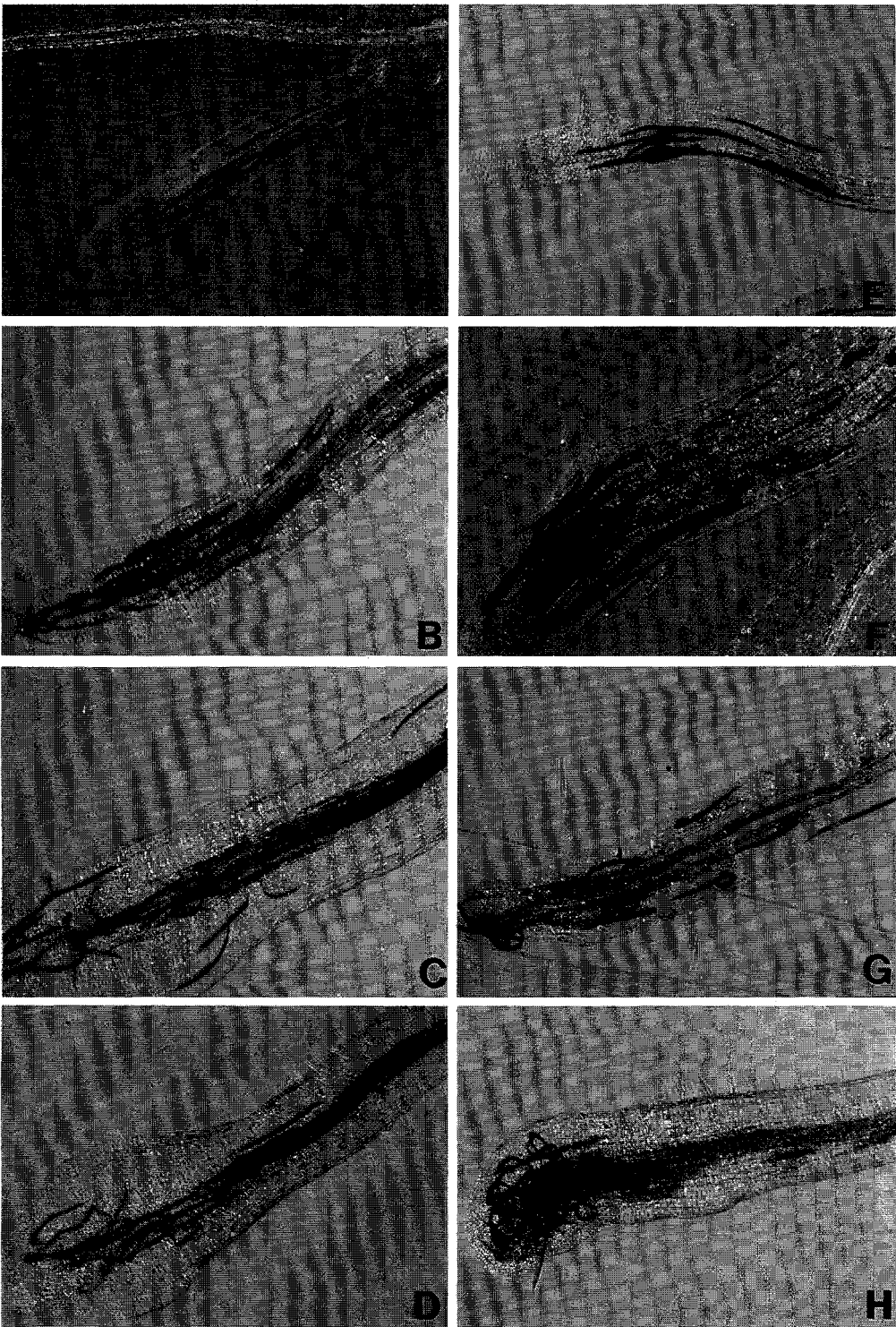


FIG. 1. Migration of *Meloidogyne incognita* second-stage juveniles (stained red) in alfalfa root tips. A–D) susceptible cultivar Lahontan at 12, 24, 48, and 72 hours post inoculation, respectively; E–H) resistant cultivar Moapa 69 at 12, 24, 48 and 72 hours post inoculation, respectively. (Scale bar = 150 μ m)

Although a few J2 were present in the root apices of Moapa 69 after 7 days, none were observed 14 or 21 days post inoculation (data not shown). Numbers of J2 present in roots of Lahontan 7 days post inoculation ranged from two to nine nematodes per root tip, most of which were noticeably enlarged and positioned along the vascular cylinder (data not shown). Females with egg masses were present in roots of Lahontan 21 days following inoculation. No differences in root necrosis were observed between inoculated plants of Lahontan and Moapa 69 at any time during the experiments.

Host gene expression: Total RNA was isolated from roots of the susceptible host at 24 hours, 72 hours, and 14 days post inoculation. These time points coincided with the presence of J2 in the root apex (24 hours), the migration of J2 into the vascular cylinder of the susceptible host (or failure to enter the vascular cylinder in the resistant host, 72 hours), and the establishment of successful feeding sites in the susceptible host (14 days). Differences in the RNA translation products between the control and infected roots were observed

as early as 24 hours post inoculation (Fig. 2). In the susceptible cultivar, most early differences in translation products 24 hours after inoculation were more pronounced 72 hours after inoculation (Fig. 3). Many novel or highly enhanced translation products in the molecular mass ranges of 55 kDa, 45 kDa, and 27 kDa were detected after 72 hours when inoculated roots were compared with control roots of susceptible Lahontan (Fig. 3A,B,E). Some translation products present in the uninoculated roots were not present or were greatly reduced in the inoculated roots (represented by arrows, Figs. 2,3). These products may represent genes that are down-regulated as a result of the infection process.

Changes in gene expression in the roots of resistant Moapa 69 also were detected 72 hours after inoculation (Fig. 4). Enhancement of translation products in inoculated roots compared to control roots occurred to a lesser extent within boxed areas of Moapa 69 analogous to similarly labeled areas shown in the 72-hour Lahontan profiles (Fig. 3). The 45 kDa protein, which was greatly enhanced in the infected

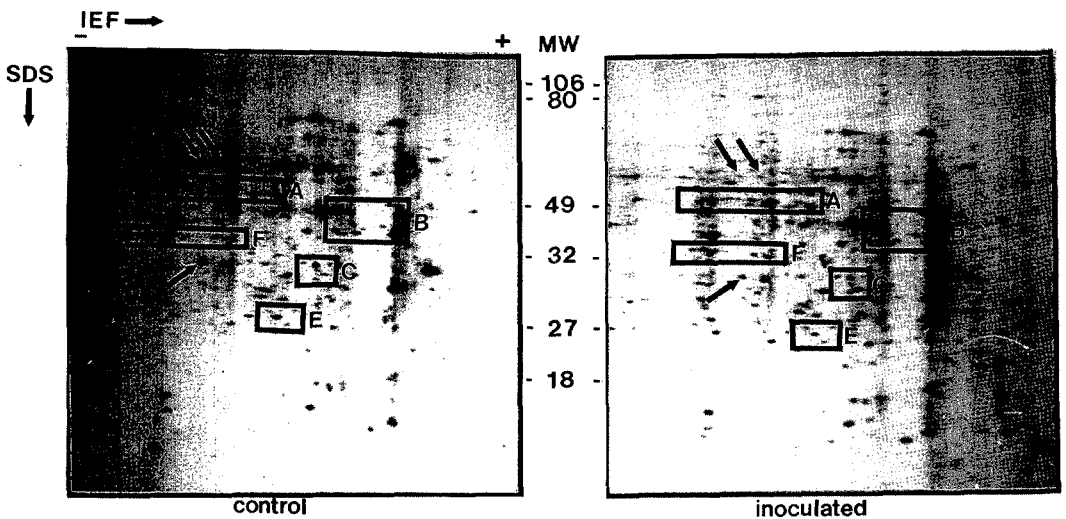


FIG. 2. Comparison of the 2D gel profiles of in vitro translation products of RNA from Lahontan alfalfa roots 24 hours after inoculation with *M. incognita* (inoculated) or treatment with distilled water (control). Boxes A,B,C,E,F (D not included) enclose corresponding regions of the gels in which translation products are enhanced or unique in inoculated roots, and are analogous to boxes with the same letters in Figs. 3 and 4. Arrows indicate translation products that are absent or reduced in inoculated roots. Molecular weights are given in thousands.

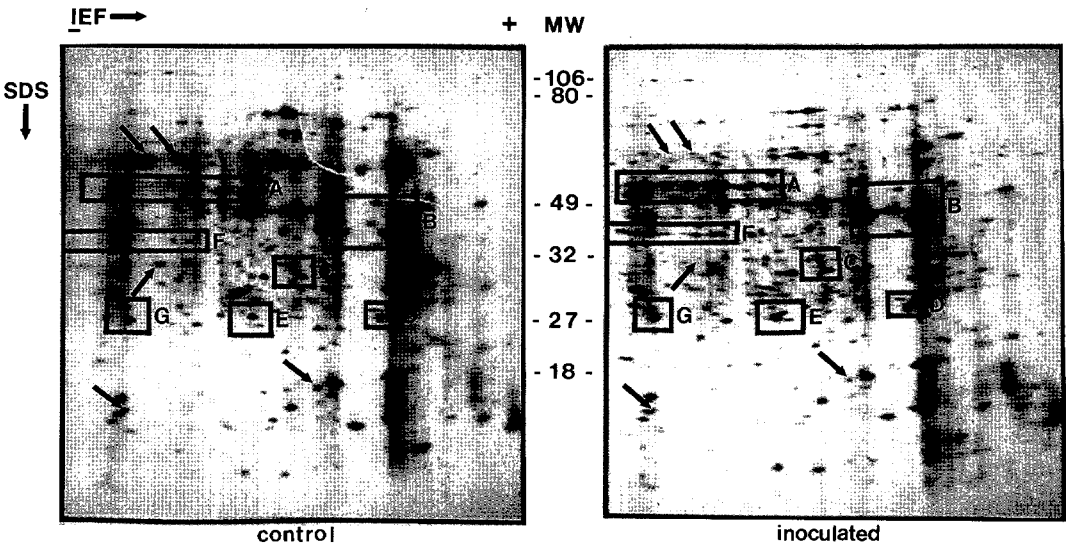


FIG. 3. Comparison of the 2D gel profiles of the in vitro translation products of RNA from Lahontan alfalfa 72 hours after inoculation with *M. incognita* (inoculated) or treatment with distilled water (control). Boxes A–G enclose corresponding regions of the gels in which translation products are enhanced or unique in inoculated roots, and are analogous to boxes with similar letters in Figs. 2 and 4. Arrows indicate translation products that are absent or reduced in inoculated roots. Molecular weights are given in thousands.

roots of the susceptible host at 72 hours post inoculation (Fig. 3B), also increased in the resistant host (Fig. 4B) in response to infection. The profile of translation products from roots of the two cultivars also included areas in which each cultivar re-

sponded uniquely to inoculation (Fig. 3D,E, arrows; Fig. 4H). When RNA was isolated from root tips of the susceptible host 14 days post inoculation, a time when successful feeding sites had formed, no difference in gene products could be de-

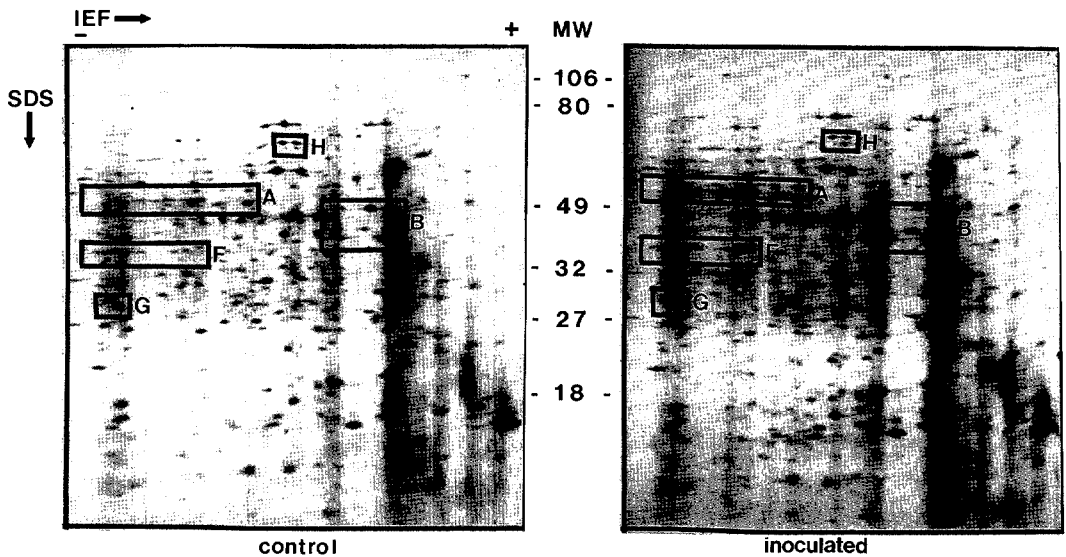


FIG. 4. Comparison of the 2D gel profiles of the in vitro translation products of RNA from Moapa 69 alfalfa roots 72 hours after inoculation with *M. incognita* (inoculated) or treatment with distilled water (control). Boxes A,B,F,G,H enclose corresponding regions in which translation products are enhanced or unique in inoculated roots, and are analogous to boxes with similar letters in Figs. 2 and 3. Molecular weights are given in thousands.

tected between control and infected roots (data not shown).

Many novel translation products were detected in gels of wounded roots, some of which co-migrated with the translation products of RNA from infected roots (Fig. 5). However, some of the translation products specific to or enhanced in the infected roots were not detected in the 2D gel profile of translation products corresponding to RNA from wounded roots (Fig. 5B–D, small arrows). Although there were similarities in some products between the wounded and the inoculated roots, other products were unique to the wounded roots (Fig. 5A–D, large arrows). The 2D

profile of wounded Moapa 69 was not included because we were interested only in determining the extent to which gene products induced by nematode infection in Lahontan might also result from wounding.

DISCUSSION

The resistance response to *M. incognita* in alfalfa occurs within 3 to 4 days after root penetration and without the development of a hypersensitive response (34). Our results indicate that this is due, at least in part, to failure of J2 to enter the vascu-

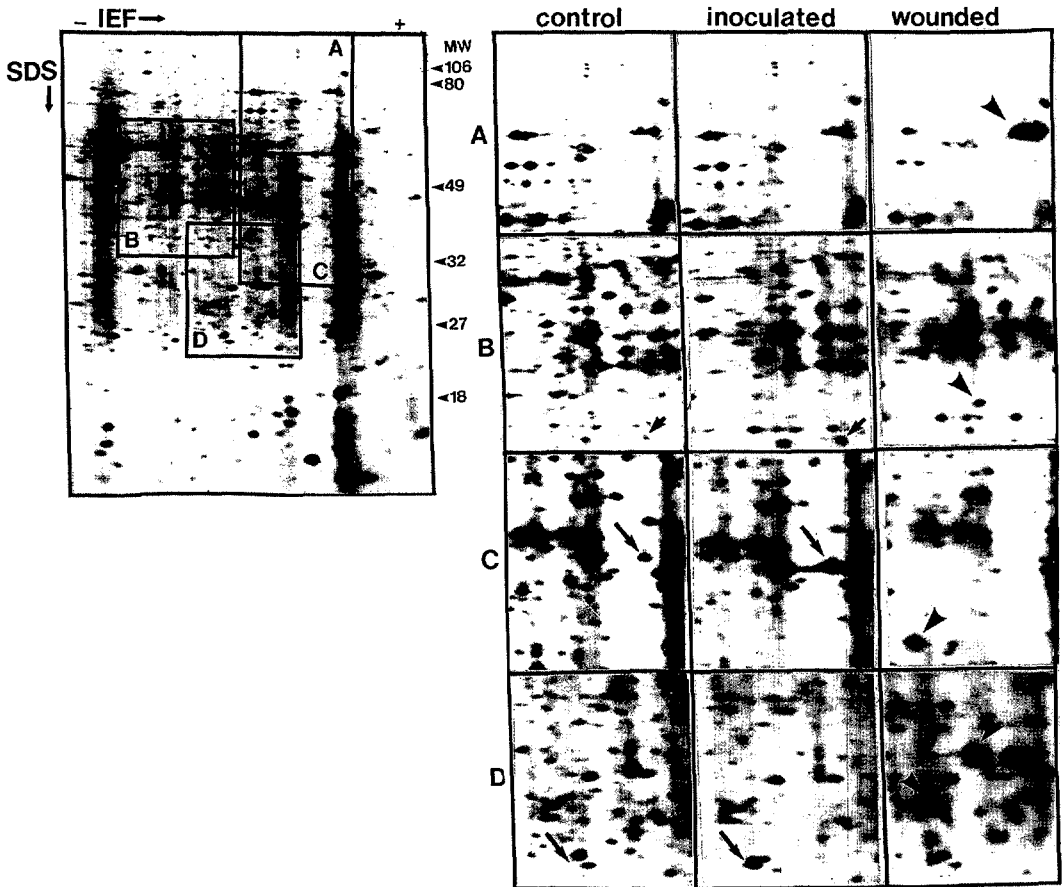


FIG. 5. Comparison of analogous regions from 2D gel profiles of in vitro translation products of RNA from Lahontan alfalfa roots 72 hours after inoculation with *M. incognita* (inoculated), treatment with distilled water (control), or mechanical wounding (wounded). Gels were partitioned similarly (boxes A–D) and boxes were enlarged for comparison across columns within rows A–D. Pairs of small arrows within a row indicate translation products unique to nematode infection. Large arrows in the last column indicate products uniquely associated with wounding. Molecular weights are given in thousands.

lar cylinder of resistant plants following migration to the root apex. In other crops where incompatibility is associated with a hypersensitive response, J2 enter the stele, then root cell necrosis occurs in or immediately adjacent to developing giant cells (13,21,24,32). In tomato plants possessing the *Mi* resistance gene, necrosis occurs within 1 or 2 days after infection and is localized around the nematode and its feeding site (32). In soybean, both a visible hypersensitive response and accumulation of the phytoalexin, glyceollin, occur inside the stele of resistant, but not susceptible, cultivars when feeding commences (23, 24). Soybean incompatibility to *M. incognita* has been linked to declining levels of superoxide dismutase in resistant plants (38). Incompatibility between the alfalfa cultivar Moapa 69 and *M. incognita* apparently occurs before J2 can enter the stele, where hypersensitive responses are normally induced, possibly explaining the absence of root necrosis in this cultivar.

Rates of infection and nematode behavior during the initial 24 hours following inoculation were similar in roots of Lahontan and Moapa 69, and agree with previous findings (34). Differences in J2 behavior between the cultivars first appeared 48 to 72 hours after infection, coinciding with the entry of J2 into the vascular cylinder of Lahontan. All subsequent observations revealed continued nematode development in Lahontan and egress or death of nematodes in Moapa 69, confirming earlier reports (34). The reason for failure of J2 to enter the vascular cylinder of Moapa 69 is unclear. We observed no microscopic evidence of physical barriers, cell necrosis, or other conditions that might inhibit movement of J2 into the developing vascular cylinder in root apices of Moapa 69. A possible explanation may be that a signal present in susceptible alfalfa induces entry of J2 into the vascular cylinder, whereas this signal is absent, reduced, or somehow masked in Moapa 69.

The most substantial changes in host gene expression between infected and uninfected roots of Lahontan occurred 72

hours after inoculation, after J2 had entered the vascular cylinder. We compared the gel profiles of infected roots of Lahontan and Moapa 69 in order to rule out the possibility that nematode RNA was responsible for changes in the 2D profile of translation products of RNA from infected roots. While root tips from both cultivars contained similar numbers of J2, major changes in gene products occurred only in Lahontan. During the initial 24 hours after inoculation, few changes in host gene expression were observed in either cultivar as J2 migrated intercellularly through cortical tissue. There is little evidence of plant injury before J2 enter the vascular cylinder (12,42).

Some of the gene products induced 72 hours after inoculation were the same as those induced by mechanical wounding. Such findings are to be expected, considering the high level of nematode infection in our study and previous observations of injury to meristematic tissue as J2 enter the developing vascular cylinder (42). Such injury may account for much of the wounding response observed. However, a few gene products were unique or greatly enhanced only in the infected roots of Lahontan. The functional role of such gene products and their relationship to the entry of J2 into the vascular cylinder are not known. Most likely the gene products are not directly involved in giant cell development or maintenance because similar changes in gene expression were not detected in giant cell-containing roots 14 days after inoculation. Failure to detect any giant cell-specific transcripts may be due in part to a dilution factor. The giant cells comprise a relatively small percentage of the total root, and the translation products corresponding to the giant cell-specific transcripts may be masked by the transcripts from the predominant root tissue. By limiting the source of RNA exclusively to giant cells, others have isolated cDNAs (41) that are specifically induced in these feeding sites. Similarly, a cDNA library to RNA was constructed from syncytia-enriched regions of potato roots and an

infection-specific cDNA associated with *G. rostochiensis* was isolated (16).

Moapa 69 appears to possess a unique type of early resistance to *M. incognita* that does not depend on a hypersensitive response. Other alfalfa cultivars exhibiting resistance to *M. hapla* may possess a similar mechanism (15). Characterization of the novel gene products specifically produced in the resistant plants between 48 hours and 72 hours post inoculation should contribute toward understanding the resistance mechanism in Moapa 69. The 2D gel profile of the translation products from root RNA of resistant plants 72 hours after inoculation did not, however, show many changes in gene expression in our study. Some of the minor changes that were seen are related to changes also observed in Lahontan following infection. Again, it is likely that our procedures were not sensitive enough to detect small changes in host gene expression. Hammond-Kosack et al. (17) also used 2D analysis of translation products to detect novel gene products in susceptible and resistant potato roots following infection with *G. rostochiensis*. Most of the early changes in gene expression in their study appeared to be a consequence of wounding, which is much more pronounced during cyst nematode invasion compared to root-knot nematode invasion (12).

Our results demonstrate the relationship between appearance of novel gene products and migration of J2 into the vascular cylinder of the susceptible host. Isolation and characterization of these genes should aid in understanding the basis for susceptibility and resistance mechanisms in plant-nematode interactions where early host resistance does not result from a visible hypersensitive response.

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