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Are Pathogenesis-Related Proteins Induced by *Meloidogyne javanica* or *Heterodera avenae* Invasion?[1]

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Abstract: Changes in root- and leaf-soluble proteins were investigated in tomato after invasion by the root-knot nematode *Meloidogyne javanica,* or in barley and wheat after invasion by the cereal cyst nematode *Heterodera avenae.* Infection of susceptible tomato plants by *M. javanica* did not cause any change in the soluble-protein composition of leaves or roots compared with uninoculated plants at an early infection stage. No pathogenesis-related proteins (chitinase, glucanase, or P-14) were induced in the leaf apoplast. Changes in leaf proteins were not observed after invasion of wheat cultivars by *H. avenae,* whereas, in barley, a few changes in intercellular leaf proteins were recorded in resistant cultivars. These changes, however, were not the same among different *H. avenae*-resistant cultivars. Protein changes were found at an early stage of infection in barley and wheat roots infected with *H. avenae,* but no difference was found between resistant and susceptible cultivars.

Key words: barley, cereal cyst nematode, chitinase, glucanase, *Heterodera avenae, Hordeum vulgare, Lycopersicon esculentum, Meloidogyne javanica,* nematode, pathogenesis-related proteins, root-knot nematode, tomato, *Triticum aestivum,* wheat.

Plants react in several different ways to defend themselves against pathogens. Secondary metabolites, such as phytoalexins and phenolic compounds, are often synthesized following pathogen invasion (Zacheo and Bleve-Zacheo, 1995), and a range of plant defense-related proteins also is induced (Bowles, 1990). Pathogenesis-related (PR) proteins, usually 10 to 40 kDa in size, are a group of host-encoded, inducible proteins whose synthesis often is associated with certain forms of resistance to pathogens and stress (Bowles, 1990; Carr and Klessig, 1989). PR proteins originally were found in tobacco plants infected with tobacco mosaic virus and have since been found in both dicots and monocots (Carr and Klessig, 1989; Cordero et al., 1992; Nasser et al., 1990; White et al., 1989). In tobacco, PR proteins are classified into five groups: PR-1 (unknown function), PR-2 (β-1,3-glucanase), PR-3 (chitinase), PR-4 (hevein-like protein), and PR-5 (thaumatin-like protein). PR-1, PR-2, and PR-3 also contain basic proteins, in addition to the acidic forms. Acidic PR proteins are strongly induced by salicylic acid (SA), whereas basic β-1,3-glucanase and basic chitinase are induced by ethylene (Meins et al., 1991). These proteins have been shown to exhibit antimicrobial activity in vitro (Ryals et al., 1996). Although the biochemical function of proteins belonging to the PR-1 group is not yet clear, they also may possess antifungal activity (Alexander et al., 1993). Accumulation of PR proteins is generally associated with increased resistance to subsequent challenge by pathogens in inoculated and uninoculated parts of the plant. This phenomenon is known as systemic acquired resistance (SAR) (Ryals et al., 1996). PR proteins are also found in uninfected plants at certain developmental stages, such as flowering (Lotan et al., 1989), and can be induced in plants by chemical elicitors such as SA and ethylene (Kessman et al., 1994). SA has been thought to play an important role in SAR because exogenous SA induces this resistance and accumulates in pathogen-infected tissue (Malamy et al., 1990; Metraux et al., 1990) and is translocated to uninfected parts of the plant (Shulaev et al., 1995). However, SA is not thought to be the signal for SAR in plants (Vernooij et al., 1994).

In plant-nematode interactions, newly synthesized proteins, including PR proteins, have been studied in potato plants after infection by the potato cyst nematodes *Glo-*

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bodera pallida and *G. rostochiensis* (Hammond-Kosack et al., 1989; Rahimi et al., 1993, 1996). In this system, some of the proteins were also induced by treating leaves with SA or aspirin (Hammond-Kosack et al., 1989; Rahimi et al., 1993). Changes in protein composition and translatable mRNA following nematode infection were more obvious in the foliage than in the roots (Hammond-Kosack et al., 1990). The activities of exoglucanase in the apoplast, endoglucanase in homogenate, and exoglucosidase in the apoplast increased in leaves of infected potato plants, whereas exoglucanase or exoglucosidase activity did not increase in infected roots (Rahimi et al., 1996). Although accumulation of these enzymes depended on the potato cultivar and on the nematode species and pathotype, the exact role or involvement of these proteins in resistant and susceptible relationships has not been clarified. In tall fescue (*Festuca arundinacea* Schreb.) infected with *Meloidogyne marylandi,* chitinase activity in the foliage was higher than that of uninfected control plants and was related to plant persistence (Roberts et al., 1992).

In the research reported herein, local and systemic induction of PR proteins was investigated in tomato (*Lycopersicon esculentum* Mill.) plants inoculated with the root-knot nematode *Meloidogyne javanica* (Treub) Chitwood, and in barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) infected with the cereal cyst nematode *Heterodera avenae* Wollenweber.

Materials and Methods

Nematodes: Second-stage juveniles (J2) of *M. javanica* were hatched from eggs extracted from tomato cv. Faculta 68 roots with a sodium hypochlorite solution (Hussey and Barker, 1973). *Heterodera avenae* pathotype Ha21 J2 were hatched from newly formed, dark-brown cysts collected from a culture maintained on wheat in the Department of Nematology at Bet Dagan. The cysts were surface-sterilized for 3 minutes with a 1% sodium hypochlorite solution mixed with 0.01% Tween 20, soaked for 10 minutes in a solution of 3% H_2O_2 mixed with 40% ethanol (1:1, v/v), and washed thoroughly with sterilized water. Surface-sterilized cysts were placed on a 60-µm-aperture nylon sieve immersed in a 20-ml bottle containing 5 ml water, and maintained at 15 °C. After ca. 2 weeks, J2 were collected daily and stored at 4 °C for no longer than 3 weeks before use.

Nematode-infected plants: Soil infecting 4-week-old Faculta 68 tomato plants was infested with *M. javanica* J2 at 200, 400, 600, 800, or 1,000/plant by injection into the soil, and kept at 27 ± 1 °C with 12 hours of light. At inoculum levels higher than 400 J2/plant, root-galling indices (0 to 5) (Kinloch, 1990) were about 4.5 to 5.0 three weeks after inoculation.

Siri (an Ha21-resistant barley cultivar), Capa (an Ha21-susceptible wheat cultivar), and partially Ha21-resistant wheat cultivars 63/1-7-15-12 (Loros) and Iskamish-K-2 were provided by the Department of Crop Husbandry and Plant Breeding, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. Near-isogenic barley lines Ingrid (Ha21-susceptible) and Siri × Ingrid[7] (Ha21-resistant) were provided by J. MacKey, Department of Plant Breeding, Swedish University of Agricultural Sciences, Uppsala, Sweden. A local Ha21-susceptible wheat cultivar, Ariel, was used as well. The barley and wheat seeds were surface-sterilized for 15 minutes in a solution of 0.1% (w/v) $HgCl_2$ and 0.01% Tween 20, and washed with sterilized water. The seeds were germinated at 20 °C on a moist filter paper in a petri dish. When the primary roots were about 1.5 cm long, 50 seedlings were planted in a plastic 150-ml pot filled with silica sand infested with 5,000 *H. avenae* J2. At this inoculum level, roots of both barley and wheat showed the typical symptoms of nematode infection (Mor et al., 1992). Seedlings in uninfested pots served as controls. After planting, the seedlings in the pot were kept at 15 ± 1 °C with 11 hours of light.

Induction of PR proteins: To induce PR proteins, tomato leaves were sprayed (1 ml/plant) with 5 mM salicylic acid (SA) (Sigma Chemical Co., St. Louis, MO) or 1 mM chloroethylphosphonic acid (ethephon) (Sig-

ma). Barley and wheat plants were sprayed with a 0.2% $HgCl_2$ solution mixed with 0.01% Tween 20.

Protein extraction: Roots or leaves of tomato, barley, or wheat were homogenized in 1 ml (per g tissue) of an acid buffer (84 mM citric acid, 32 mM Na_2HPO_4, and 14 mM 2-mercaptoethanol, pH 2.8) or a neutral buffer (50 mM Tris-HCl, 14 mM 2-mercaptoethanol, and 2 mM EDTA, pH 7.5) with an ice-chilled mortar and pestle. The homogenate was centrifuged at 15,000*g* (20 minutes, 4 °C), and the supernatant was stored at −20 °C until used.

Intercellular tomato-leaf proteins were extracted according to the method of DeWit and Spikman (1982) with some modifications. Leaves were vacuum-infiltrated for 5 minutes with chilled distilled water, blotted on a filter paper, and rolled in a polyethylene sheet. The rolled leaves were inserted in a 10-ml plastic syringe equipped with a 1.5-ml Eppendorf tube set in the bottom of a 50-ml tube. The whole system was centrifuged at 500*g* (5 minutes, 4 °C). The intercellular washing fluid (IWF) collected in the Eppendorf tube was further centrifuged at 15,000*g* (20 minutes, 4 °C), and the supernatant was stored at −20 °C until used. Intercellular proteins of barley and wheat leaves were extracted similarly, except that the leaves were cut into 5-cm-long segments, washed in cold distilled water, and vacuum-infiltrated with a basic buffer (50 mM Tris-HCl and 1 mM EDTA, pH 8.5). Protein concentration was determined with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin (Sigma) as a standard (Bradford, 1976).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting: Protein extracts (ca. 10 µg each) were mixed (1:1, v/v) with sample buffer (125 mM Tris-base, 20% [v/v] glycerol, 2% [w/v] SDS, 2% [v/v] 2-mercaptoethanol, and 0.02% [w/v] bromphenol blue, pH 6.8), boiled for 5 minutes, loaded onto a 1.5-mm-thick 3.9% stacking gel, and fractionated through a 12% separating gel in a Mini Protean II apparatus (Bio-Rad). The separated proteins were stained with Coomassie Blue G 250 (Serva, Heidelberg, Germany) (Neuhoff et al., 1988) or transferred to a nitrocellulose membrane with a Bio Trans Midi (Gelman Sciences, Ann Arbor, MI). Antisera against tobacco acidic chitinase, tobacco acidic PR-1, tobacco acidic glucanase, and tomato P-14 were used to detect PR proteins (Cohen, 1994). Immunoblots were visualized with the ECL reagent kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The antisera were provided by R. Fluhr, Department of Plant Genetics, Weizmann Institute of Science, Rehovot, Israel.

Results

PR-protein induction in tomato: The invasion of tomato roots by *M. javanica* J2 did not change the acid-soluble-protein composition of homogenized tomato leaves or their IWF, at levels of up to 1,000 J2/plant, 14 days after soil infestation (DAI) (Fig. 1). In contrast, treatment with SA or ethephon induced new protein bands in acid-homogenized leaves (16 kDa in both treatments and 34 kDa in ethephon-treated plants) (Fig. 1A) and in the leaf IWF (31 kDa in both treatments) (Figs. 1B,2). Chitinase, glucanase, and P-14 were not detected on an immunoblot of the IWF from *M. javanica*-infected tomato plants, although chitinases (26 and 31 kDa), glucanase (31 kDa), and P-14 (14 kDa) were induced in leaves sprayed with 5 mM SA (chitinase and glucanase) or 1 mM ethephon (all three PR proteins) (Fig. 2). In nematode-infected tomato roots, neither acid- nor neutral-soluble protein compositions were changed relative to roots of control-plants 4 DAI, and immunoblotting did not detect any chitinase, glucanase, or P-14 (not shown).

PR-protein induction in barley and wheat: Proteins of Ingrid leaf IWF were not changed by infection, but those from the *H. avenae*-infected barley cultivars Siri and Siri × Ingrid[7] differed from those of their uninfected counterparts, particularly with respect to the 27-kDa chitinase band (Fig. 3B) and proteins of 28, 33, and 35 kDa (Fig. 3A). A 43-kDa protein band in Siri was sup-

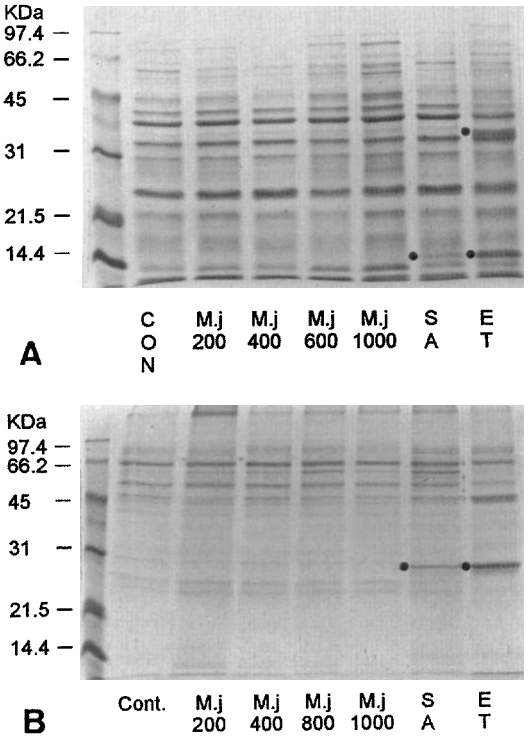

FIG. 1. SDS-PAGE of proteins extracted from tomato plants. Tomato plants were grown in soil infested with 200, 400, 600, 800, or 1,000 second-stage juveniles of *Meloidogyne javanica* (M.j.) per plant, or sprayed with 5 mM salicylic acid (SA) or 1 mM ethephon (ET). Plants in uninfested soil were used as a control (Cont.). Proteins were extracted from same-age plants, 3 days after chemical treatment or 14 days after soil infestation. Induced bands are indicated with dots to the left of their respective lanes. A) Leaf homogenate extracted at pH 2.8. B) Intercellular washing fluid. Molecular-size standards are on the left.

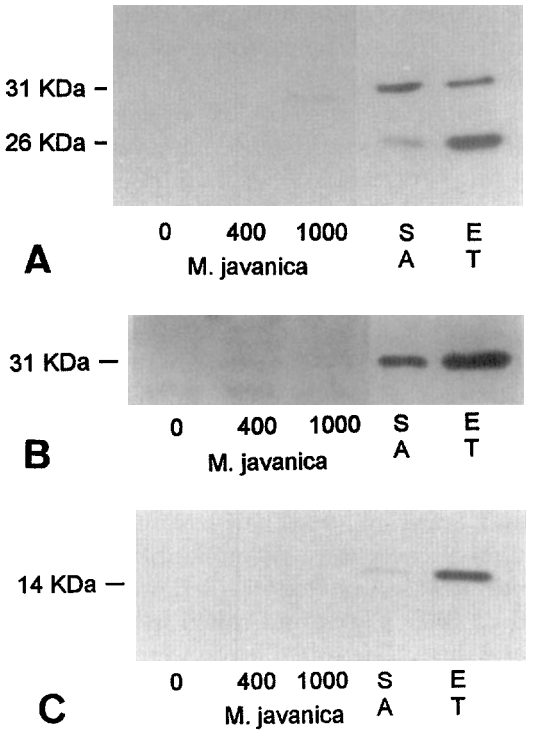

FIG. 2. Immunoblot analysis of intercellular washing fluid (IWF) from tomato leaves. Tomato plants were grown in soil infested with 400 or 1,000 second-stage juveniles of *Meloidogyne javanica* per plant, or sprayed with 5 mM salicylic acid (SA) or 1 mM ethephon (ET). IWF was taken from same-age plants, 3 days after chemical treatment or 14 days after inoculation, fractionated by SDS-PAGE, and immunoblotted with A) antiserum raised against tobacco acidic chitinase. B) antiserum raised against tobacco acidic glucanase. C) antiserum raised against tomato P-14. Numbers on the left represent the molecular sizes.

pressed following infection. $HgCl_2$-treated plants exhibited several changes in IWF proteins, mainly those in the range of 28 to 40 kDa, and at 15 and 16 kDa (Fig. 3A). One chitinase band (27 kDa) was detected in the IWF from leaves of control barley cultivars Ingrid and Siri × Ingrid[7], but the band in Siri × Ingrid[7] weakened 10 DAI (Fig. 3B). Chitinase was not detected in the IWF of uninoculated Siri but was observed 14 DAI (Fig. 3B). Neutral and acid extracts of homogenized leaves of the barley cultivars exhibited no differences in protein composition between *H. avenae*-infected and uninfected plants on a stained gel from 5 to 20 DAI (not shown).

Protein profiles of leaf IWF from *H. avenae*-infected wheat cultivars did not differ from those of their uninfected counterparts, but leaf-protein profiles from $HgCl_2$-treated wheat exhibited some differences, mainly in the proteins migrating at 16 to 44 kDa (Fig. 4A). In the leaves of the $HgCl_2$-treated plants, three chitinase bands (27, 31, and 34 kDa) were detected in the IWF but not in the IWF from leaves of *H. avenae*-infected or uninfected plants (Fig. 4B). Acid-extracted, homogenized wheat leaves of Capa, Loros, and Iskamish-K-2 exhibited no differences in protein composition among uninfected, *H. avenae*-infected, and $HgCl_2$-treated plants on a stained gel 14 DAI (not shown).

Several changes in root-protein composi-

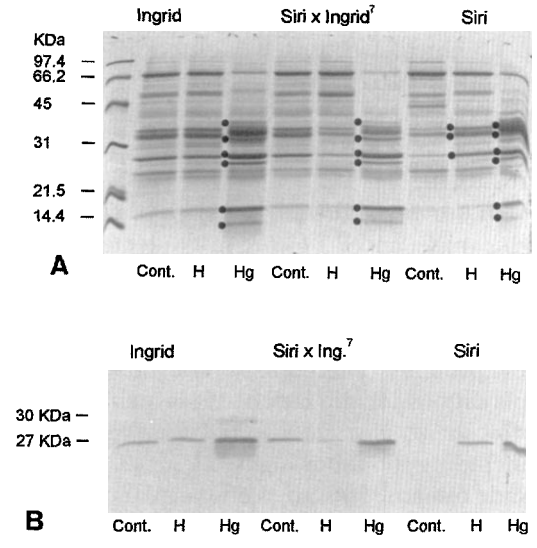

FIG. 3. Intercellular washing fluid (IWF) from leaves of the barley cultivars Ingrid, Siri × Ingrid[7], and Siri. Barley plants were grown in soil infested with 100 second-stage juveniles of *Heterodera avenae* per plant (H), or sprayed with a 0.2% $HgCl_2$ solution (Hg). IWF was taken from same-age plants, 3 days after spraying or 14 days after inoculation. Plants grown in uninfested soil were used as a control (Cont.). Strengthened or induced bands are indicated with dots to the left of their respective lanes. Numbers on the left represent the molecular sizes. A) SDS-PAGE. B) Immunoblot with antiserum raised against tobacco acidic chitinase.

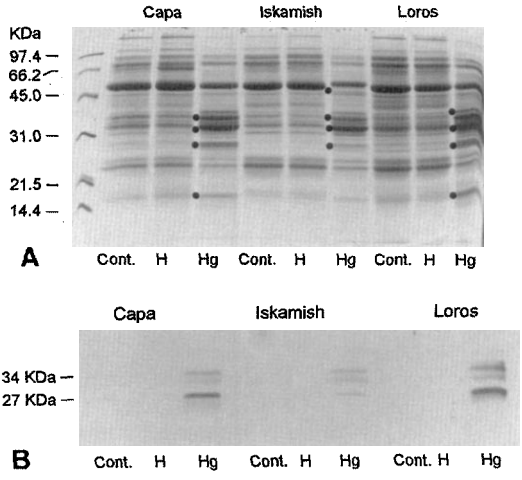

FIG. 4. Intercellular washing fluid (IWF) from leaves of the wheat cultivars Capa, Iskamish-K-2, and Loros. Wheat plants were grown in soil infested with 100 second-stage juveniles of *Heterodera avenae* per plant (H), or sprayed with a 0.2% $HgCl_2$ solution (Hg). IWF was taken from same-age plants, 3 days after spraying or 14 days after inoculation. Plants grown in uninfested soil were used as a control (Cont.). Strengthened or induced bands are indicated with dots to the left of their respective lanes. Numbers on the left side represent the molecular sizes. A) SDS-PAGE. B) Immunoblot with antiserum raised against tobacco acidic chitinase.

tion were observed in acidic extracts of homogenized barley and wheat roots infected with *H. avenae* J2, relative to uninoculated root proteins, 4 DAI (Fig. 5). In the barley cultivars, a protein band of 97 kDa was stronger in the *H. avenae*-infected roots, whereas a band of 86 kDa was weaker (Fig. 5A). Protein bands of 34 and 47 kDa were induced, and bands of 76, 70, and 31 kDa were strengthened, whereas bands of 50 and 40 kDa were weakened (Fig. 5A). In the wheat cultivars, changes in root proteins after nematode infection were less obvious: 97, 66, and 31-kDa protein bands were stronger in *H. avenae*-infected roots, whereas bands of 86 and 40 kDa were weaker (Fig. 5B). The changes described here were the same in susceptible and resistant barley and wheat cultivars. PR-1 was not detected in the leaves or roots of either infected or uninfected wheat or barley cultivars 7 or 14 DAI (not shown).

DISCUSSION

Most studies on PR proteins have been performed with foliage of dicots, although PR proteins from monocots (Bryngelsson et al., 1988; Cordero et al., 1992; Nasser et al., 1990; Roberts et al., 1992) and those in the roots of monocots and dicots have also been studied (Benhamou et al., 1991; Tahiri-Alaoui et al., 1993). The induction of these proteins by pathogens or chemicals is well documented (Brederode et al., 1991; Carr and Klessig, 1989). Generally, acidic PR proteins are present at low levels in healthy plants and are induced extracellularly after pathogen invasion. SA is a strong inducer of acidic PR proteins. In contrast, basic PR proteins are present at high levels in healthy plant roots, are located intracellularly in the vacuoles, and are also induced in leaves by stress signals or ethylene (Memelink et al., 1990; Ward et al., 1991). In the present study, dicots and monocots were used to analyze PR-protein induction following nematode infection. There are some differ-

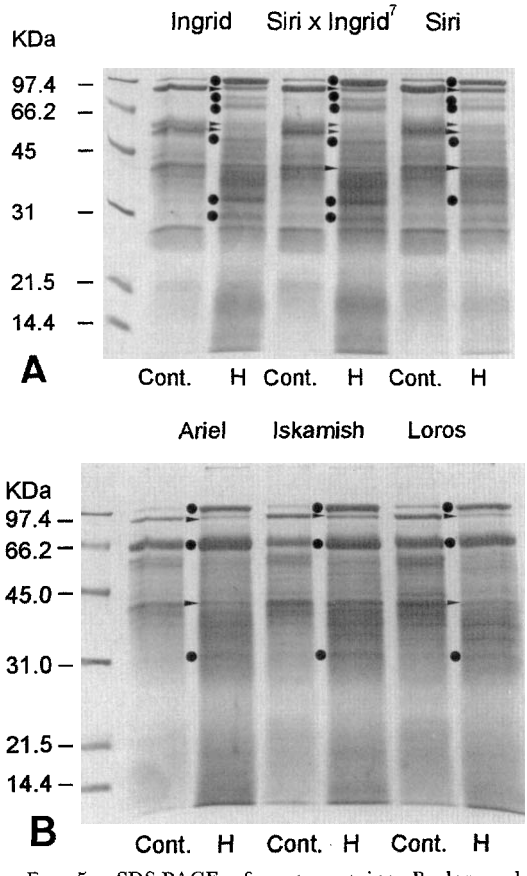

FIG. 5. SDS-PAGE of root proteins. Barley and wheat seedlings were grown in soil infested with 100 second-stage juveniles of *Heterodera avenae* per seedling (H). Proteins were extracted at pH 2.8, 4 days after inoculation: Plants grown in uninfested soil were used as a control (Cont.). Induced or strengthened bands are indicated by dots, and weakened bands by arrows, to the left of their respective lanes. A) Root proteins of the barley cultivars Ingrid, Siri × Ingrid[7], and Siri. B) Root proteins of the wheat cultivars Ariel, Iskamish-K-2, and Loros. Molecular-size standards are on the left.

ences between these plant types with regard to PR-protein induction; for example, SA and ethephon are not effective PR-protein elicitors in barley and wheat (Fischer et al., 1989; unpublished data).

Changes in protein composition of potato plants infected with the potato cyst nematodes *G. pallida* and *G. rostochiensis,* in both compatible and incompatible plant-nematode interactions, were induced locally and systemically, and changes in leaf-protein composition were similarly influenced by aspirin, a known inducer of PR proteins (Hammond-Kosack et al., 1989; Rahimi et al., 1993). In subsequent work, activities of β-1,3-glucanase and β-D-glucosidase were found to increase in leaves of infected potato plants, whereas β-1,3-glucanase activity did not increase in infected roots (Rahimi et al., 1996).

In our work, the acidic forms of chitinase, glucanase, and P-14 were not detected in the leaves or roots of tomato plants infected with *M. javanica,* whereas chemical treatments with SA or ethephon induced these PR proteins. There are several possible explanations for the lack of PR-protein induction in *M. javanica*-infected tomato plants, including the following: i) *M. javanica* J2 invade tomato roots intercellularly, and the resultant wounding that could trigger defense-related genes is less severe than that caused in potato roots by potato cyst nematodes (Hansen et al., 1996); ii) in contrast to a syncytium, the feeding site of cyst nematodes, the feeding site of root-knot nematodes (giant cells) is initiated without the cell-wall dissolution that can trigger a defense reaction; iii) nematode infection may downregulate a systemic signal leading to PR proteins in leaves (Goddijn et al., 1993); and iv) physiological processes in tomato plants may differ from those in potato plants. For example, ethylene, an inducer of PR proteins, is synthesized upon nematode infection (Glazer et al., 1983) and general stresses in plants; however, it is only a weak inducer of PR proteins in tomato plants (Cohen et al., 1994).

No change in protein composition was detected in the *M. javanica*-infected tomato roots 4 DAI, and there was no evidence of a particular PR protein. The absence of such a change may be associated with intercellular migration. An interesting piece of evidence is that greater changes in translatable mRNA have been found in leaves than in roots of potato plants infected with *G. rostochiensis* (Hammond-Kosack et al., 1990).

Changes in protein composition caused by *H. avenae* infection were recorded in the IWF of resistant barley cultivars, but these changes differed among the cultivars. The constitutive chitinase found in Ingrid and Siri × Ingrid[7] was depressed in *H. avenae*-infected Siri × Ingrid[7], whereas nematode

infection and $HgCl_2$ induced the chitinase in Siri. This result is in contrast to that in potato plant-nematode interactions, in which changes in the host protein were greater in compatible interactions than in incompatible ones (Rahimi et al., 1993). On the other hand, such changes were not observed in the IWF from the wheat cultivars. In tall fescue, a perennial grass, chitinase was systemically expressed in the foliage of plants infected with *M. marylandi* but not in the roots (Roberts et al., 1992).

In *H. avenae*-infected roots of barley and wheat, several changes were observed at an early stage of infection 4 DAI: a 97-kDa band became stronger and an 86-kDa band became weaker in both barley and wheat cultivars, and new 34- and 47-kDa bands were induced in barley. Invasion of potato roots by *G. pallida* also induced proteins with molecular masses (70 and 82 kDa) larger than those of common PR proteins (Rahimi et al., 1993). These changes at an early stage of infection by *H. avenae* may be a response to the migration of nematodes that destroy cells and cause necrosis in the roots, and to feeding-site induction. A syncytium is formed by the fusion of several cells followed by the dissolution of their cell walls. Such dissolution also may cause plant-defense response. A change in host gene expression early in the infection also has been recorded in tobacco roots infected with the tobacco cyst nematode *G. tabacum* during J2 migration, as well as with *M. javanica* during the first steps of feeding-site initiation (Niebel et al., 1993). In transgenic potato roots, the wound-inducible transgene *wun1-udiA* was extensively expressed during intracellular migration by *G. pallida*, probably due to wounding of root cells (Hansen et al., 1996). However, we do not know whether the changes recorded in cereal root proteins are a specific response to nematode infection or are caused by general stress or wounding. Further investigation is needed to clarify the role of the root proteins.

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