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Enzymes of the Phenylpropanoid Pathway in Soybean Infected with *Meloidogyne Incognita* or *Heterodera Glycines*[1]

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Abstract: Transcription of genes encoding several enzymes and the activity of some of these enzymes of the phenylpropanoid pathway leading to synthesis of chemical and physical barriers for defense of plants against root pathogens was estimated in susceptible and resistant soybean infected with *Heterodera glycines* race 3 or with *Meloidogyne incognita* race 3. Transcription of genes encoding phenylalanine ammonia lyase (PAL) and the activity of this enzyme increased in resistant, but not susceptible, soybean cultivars after nematode infection. Likewise, transcription of the gene encoding 4-coumaryl CoA ligase and activity of this enzyme were enhanced in resistant, but not susceptible, soybean cultivars after nematode infection. Activity of PAL decreased in susceptible soybean after *H. glycines* or *M. incognita* infection. Transcription of enzymes later in the phenylpropanoid pathway leading to glyceollin synthesis increased in both resistant and susceptible soybean in response to nematode infection; the increase was greater in resistant cultivars. These results suggest possible reasons for the rapid induction of glyceollin synthesis immediately after infection of resistant soybean cultivars with *H. glycines* or *M. incognita* and the failure of this response in infected, susceptible soybean cultivars. Nematode infection had no effect on the activity of enzymes in the branch of the pathway leading to lignin synthesis.

Key words: chalcone isomerase, chalcone synthase, cinnamyl alcohol dehydrogenase, glyceollin, *H. glycines*, *M. incognita*, nematode, phenylpropanoid, resistance, root-knot nematode, soybean, soybean cyst nematode.

Many plants respond to pathogens by eliciting a hypersensitive reaction (HR) at the site of infection and subsequently, a systemic resistance response often develops (17,21,22). Thus, in some plant-pathogen interactions, like those of tobacco mosaic virus or tobacco necrosis virus or certain fungi with tobacco, the response not only becomes systemic but systemically acquired resistance (SAR) results, protecting against additional infection (18). These SAR responses usually are not specific for a given pathogen but may have some specificity for pathogen type (18). The initial response at the site of infection often is one attempting to limit movement of the pathogen through localized necrosis; strengthen cell walls by deposition of lignin or suberin or of hydroxyproline-rich or hydroxyglycine-rich glycoproteins; or produce phytoalexins, active oxygen species, or pathogenesis-related proteins (PR) that prevent establishment of the pathogen (17).

Synthesis of flavonoid or isoflavonoid phytoalexins, deposition of lignin or cell wall-bound phenolics, and synthesis of other defense chemicals via the phenylpropanoid pathway often are characteristic of both the localized HR and the SAR (17,18, 21). The phenylpropanoid pathway is a multi-branched pathway leading to synthesis of flavonoids, isoflavonoids, coumarins such as psoralen, soluble esters such as chlorogenic acid, suberin, lignin, and other wall-bound phenolics used in plant defense to pathogens and to environmental assault. This pathway is initiated by the reaction in which phenylalanine is deaminated to transcinnamic acid (Fig. 1) catalyzed by phenylalanine ammonia lyase (PAL). Next, transcinnamic acid is hydroxylated to 4-coumaric acid catalyzed by coumarate 4-hydroxylase (10). The committed reaction of this pathway occurs in the next reaction, in which 4-coumaric acid is esterified via a thiol ester with coenzyme

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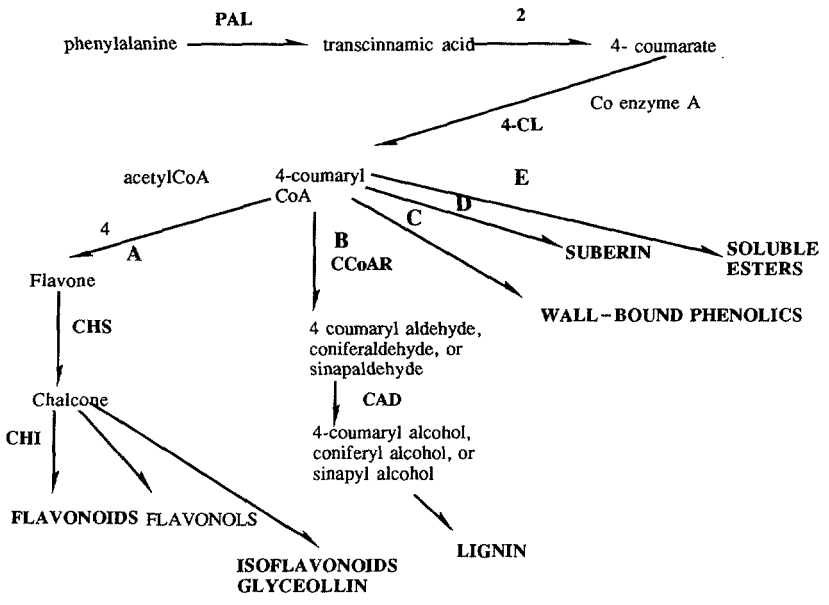

PAL: Phenylalanine ammonia lyase
2: Cinnamate-4-hydroxylase
3: 4-coumarate coa ligase
4: Acetyl coa carboxylase
CHS: Chalcone(flavone) synthase
CHI: Chalcone isomerase
CoCAR: Cinnomyl-CoA:NADP oxidoreductase (cinnomyl coa Reductase)
CAD: Cinnomyl alcohol:NADP oxidoreductase (cinnomyl Alcohol dehydrogenase)
A: flavone and isoflavonoid pathway, B: lignin pathway, C: wall-bound phenolic pathway, D: suberin pathway, E: soluble esters (e.g., Chlorogenic acids)

FIG. 1. Outline of the various branches of the phenylpropanoid pathway of secondary metabolism in plants leading to production of macromolecules involved in resistance to biotic and abiotic stress (11). *Key enzymes:* 1. Phenylalanine ammonia lyase; 2. Cinnamate 4-hydroxylase; 3. 4-Coumarate Co A ligase; 5. Chalcone synthase; 6. Chalcone isomerase; 7. Cinnamyl CoA:NADP oxidoreductase; 8. Cinnamyl alcohol:NADP oxidoreductase. *Pathway branches:* A. Flavone and flavonol glycosides; B. Lignin; C. Cell wall-bound phenolics; D. Suberin; E. Soluble esters.

A catalyzed by 4-coumarate:CoA:ligase (10).

Phenylalanine ammonia lyase activity increased in roots of *Lycopersicon esculentum* cv. Nematex infected with *M. incognita* and grown at 27 C, but no such increase occurred when this cultivar of tomato was infected with *M. incognita* and grown at 32 C (3). Thus, Nematex tomato is resistant to *M. incognita* at the lower temperature and susceptible at the higher temperature (3). Phenylalanine ammonia lyase activity is suppressed in the compatible interaction between *M. incognita* and tomato at 32 C (3). The activity of this enzyme increased in susceptible potato cultivars infected with *H. rostochiensis*, but decreased in susceptible potato cultivars infected with *M. hapla* (8).

Soybean responds to both *M. incognita* and *H. glycines* (soybean cyst nematode, SCN) by synthesis of the isoflavonoid phytoalexin glycellolin (13–16). This biologically active molecule accumulates in the stele of soybean cultivars resistant to *M. incognita* within 3 days of infection but

does not accumulate as quickly in susceptible cultivars (15,16). Glyceollin I accumulates immediately around the head of the nematode in roots of the resistant Centennial soybean 2 to 3 days after infection with *H. glycines* (13). Glyceollin III accumulates in smaller quantities 4 days later than glyceollin I, and glyceollin II does not accumulate in infected roots of Centennial soybean. Induction of feeding site differentiation by *M. incognita* or SCN has been associated with the accumulation of glyceollin (13). Phenylpropanoid phytoalexins also are induced in other nematode-plant interactions. For example, lima bean is resistant to *Pratylenchus scribneri,* but snapbean is not. Infection of lima bean with this nematode induces toxic accumulations of coumestrol and other coumestans, but no such synthesis occurs in the susceptible snapbean (22). Wall-bound phenolics also have been implicated in plant resistance to some nematodes (27).

When root-knot or cyst nematodes infect plants, the nematode first migrates in the host root tip before becoming sedentary and establishing a permanent feeding site. Considerable disruption of cellular integrity occurs as the juvenile nematodes migrate into the plant and back out to form a feeding site of a syncytium or giant cell. Observations of increased PAL activity and glyceollin synthesis in resistant soybean cultivars after infection with *M. incognita* or SCN (13–16) suggest that disruption of cellular integrity at the infection site and initiation of a nematode feeding site may induce a local response. In turn, this response may induce the phenylpropanoid pathway for the timely synthesis of flavonoids, lignins, and phenolics to protect the resistant soybean from nematode infection or it may induce synthesis of salicylic acid as a signal transducer to induce defense mechanisms (26,29). The phenylpropanoid pathway also may be induced in susceptible plants, but the induction may occur only after the nematode has effectively developed a feeding site. We have assessed the induction of synthesis and activity of enzymes of the phenylpropanoid pathway in susceptible and resistant soybean to better understand how regulation of these enzymes may determine the ability of resistant soybean to respond to nematode infection and how this regulation may fail in the compatible interaction.

Materials and Methods

Soybean cultivars: Centennial, Forrest and Hartwig cultivars, resistant to SCN race 3 and to *M. incognita* race 3 and Davis and Essex cultivars, susceptible to these nematodes (1), were used. Hartwig does support moderate reproduction of *M. incognita.* Seeds were surface-sterilized with successive washes for 4 minutes in 95% ethanol, for 10 minutes in 10% sodium hypochlorite, and for 10 minutes in a 0.01% sterile benamyl solution. The seeds were germinated and grown in sterile silica sand with a 12-hour photoperiod, relative humidity of 75 ± 5% and at 27 ± 3 C. Seedlings were watered daily with sterile distilled water, and at day 3 the seedlings were watered with a sterile solution of 1% Osmocote plant food (14-14-14).

Nematodes: Stock populations of *M. incognita* race 3 were maintained on *Lycopersicon esculentum* cv. Rutgers in a growth chamber with a 12-hour photoperiod, light intensity of 21.6 Klux, relative humidity of 75 ± 5%, at 25 ± 3 C. Plants were infected in the two-leaf stage with an axenized population of 4,000 ± 300 second-stage juveniles (J2) per plant (14). Tomato plants were watered daily and received a solution of 1% Osmocote Plant Food (14-14-14) weekly. After 60 days, the plants were harvested and the roots rinsed in sterile distilled water. The roots were chopped and *M. incognita* eggs recovered (2). The eggs were washed briefly in sterile distilled water and suspended over chlorine free water on a 38-μm opening (400-mesh) sieve. Those juveniles that hatched on days 1 and 2 were used to reinfect plants for a stock population. Beginning on day 3, J2 were collected by centrifugation and surface-sterilized in 0.1% chlorhexidine diacetate for 4 minutes. After two rinses in sterile

distilled water, the juveniles were used to infect soybean seedlings in the two-leaf stage. *Heterodera glycines* race 3 populations were maintained on susceptible Essex in the greenhouse (1). Seedlings were infected in the two-leaf stage, and after 60 days the plants were harvested and the roots were washed briefly in distilled water and then chopped. Mature cysts were washed from the roots onto sieves with 250-mm openings (60-mesh). The cysts were rinsed briefly in sterile distilled water and broken with a rubber policeman. The freed eggs were surface-sterilized (as above) and suspended over sieves with 38-μm openings (400-mesh) for hatching. Juveniles were collected beginning at 3 days of hatching and were surface-sterilized. They were then used to infect soybean seedlings at the two-leaf stage. The soybean seedlings were grown aseptically in Magenta boxes (Sigma Chemical Co., St. Louis, MO).

Seedling infections: Davis and Centennial seedlings, in the two-leaf stage, were inoculated with 6,000 ± 550 surface-sterilized J2 of *M. incognita* race 3. Standard procedures were used to inoculate Essex, Forrest, and Hartwig seedlings in the two-leaf stage with 4,000 ± 650 J2 of *H. glycines* race 3. Infected seedlings were maintained axenically (as described above) with controlled conditions of light, relative humidity, and temperature. Control seedlings were wounded by cutting the roots of the seedlings in the two-leaf stage with a scalpel. Root tissue was examined microscopically in representative plants for infection at the time of harvesting.

RNA extraction: Seedlings were harvested and the root tissue frozen immediately in liquid nitrogen and stored at −80 C. Frozen root tissue was mixed with solid CO_2 and ground to a fine powder in a Krups coffee mill. One hundred milligrams of root tissue powder was homogenized in 2 ml of ice-cold RNAzol Blue B (Biotex Laboratories, Houston, TX), and 200 μl of $CHCl_3$ was added. After 5 minutes at 4 C, the phases were separated by centrifugation and an equal volume of ice-cold isopropanol was added to the aqueous phase. RNA, precipitated from this phase by incubation for 15 minutes at 4 C, was concentrated by centrifugation at 12,000*g* and washed with cold 75% ethanol. The RNA then was suspended in diethyl pyrocarbonate (DEPC)-treated distilled water, and the RNA concentration was determined by the A_{260}/A_{280} ratio (24). All glassware and plasticware were treated with DEPC.

DNA probes: pPAL5 (6) was used to probe for PAL mRNA. The pPAL5 is a 1,750-bp fragment of *Phaseolus vulgaris* genome containing the 1,520-bp opening reading frame for the PAL-translated sequence. Plasmid pCHS1 was a 1,400-bp fragment of genomic DNA containing the 1,225-bp chalcone synthase (CHS) coding sequence (23) used as a probe for chalcone synthase mRNA. The 1930 bp cDNA coding sequence for coumarate-4-CoA ligase from parsley was in plasmid pUV (11). To recover the respective gene fragments, pPAL5 was digested with Pst-1, and plasmids pCHS1 and pUV were digested with EcoR1 (24). The plant genomic DNA inserts were separated from plasmid DNA by electrophoresis on 1.2% agarose gel; the desired fragments were recovered by electro-elution and then suspended in 5 mM Tris:EDTA, pH 8.0 (24).

Northern hybridization: Total cellular RNA was denatured in 20 μl 20 × SSC (3 M NaCl/0.3 M sodium citrate, pH 7.0 with 1M HCl), 40 μl deionized formaldehyde, and 200 μl deionized formamide at 60 C for 3 minutes. After rapid cooling on ice, the denatured RNA was diluted in a solution of 5 μl 10X MOPS ([3-(N-morpholino)-propanesulfonic acid]) buffer (24), 8.75 μl of 37% formaldehyde, and 25 μl deionized formamide and DEPC-treated distilled water to a final volume of 50 μl. Aliquots containing from 0.1 to 1 μg denatured RNA were quantitatively blotted onto MagnaGraph nylon membranes (24). The dot blots were prehybridized in 2.5 ml of 20% sodium dodecyl sulfate/1 mM phosphate buffer/500 mM EDTA/10 mg per ml bovine serum albumin, at pH 7.0 for 30 to 120 minutes and at 42 C. The

DNA probes, isolated as above, were radioactively labeled by nick translation with $dCTP^{32}$, and were denatured for 10 minutes at 100 C. After rapidly cooling on ice, an aliquot of the probe was added quantitatively to the prehybridization reactions. Hybridization was at 54 C for 18 hours. The dot blots then were washed twice for 15 minutes each with 2× SSC/0.1% SDS at room temperature and then twice for 30 minutes each with 0.1% SSC/0.5% SDS at 56 C and exposed to Kodak XOmat XRA-5 film at −80 C with Dupont intensity screens. Exposure time was adjusted for maximum development within the linear exposure range of the film and was maintained constant among different experiments (24). Autoradiograms were scanned densitometrically with a Model 620 Biorad video densitometer, and a relative concentration of mRNA hybridizing to a given DNA probe was estimated based on densitometric optical density per μg RNA applied to the individual blot. Total cellular RNA hybridized to soybean ribosomal RNA was used as a control. Each hybridization was repeated in triplicate, and all RNA estimates were calculated as the mean optical density/μg RNA ± standard error.

Enzyme activities (Phenylalanine ammonia lyase): Soybean roots were harvested and immediately frozen in liquid nitrogen. The tissue then was mixed with solid CO_2 and ground to a fine powder in a Krups coffee mill. One gram of root tissue powder was homogenized in 4 ml of 50 mM Tris Cl, 0.8 mM 2-mercaptoethanol, pH 8.8 containing 400 mg of Polyclar AT to remove flavonoids and polyphenols. The homogenate was centrifuged for 10 minutes at 5,000*g* and then for 30 minutes at 16,000*g* at 4 C. Protein concentration was estimated and an aliquot of the supernatant was added to a 2-ml reaction mixture containing 2.5 mM L-phenylalanine, 25 mM Tris-Cl pH 8.8. The mixture was incubated at 40 C, with aliquots taken at 30-minute intervals, and PAL activity estimated by measuring the conversion of L-phenylalanine to transcinnamic acid as the change in absorbance at 290 nm (19). Control reactions lacked either L-phenylalanine or an aliquot of the crude homogenate. All assays were repeated at least six times and results were reported as mean ± SEM.

Cinnamoyl CoA reductase and cinnamyl alcohol dehydrogenase: Cinnamoyl-CoA reductase (CCoAR) and cinnamyl alcohol dehydrogenase (CAD) activity were determined as described by Sarni et al. (25). One gram of soybean root tissue powder was homogenized in 10 ml of buffer (100 mM Tris-Cl, pH 7.5, 15 mM 2-mercaptoethanol, 0.5% polyethylene glycol and 0.67 g Polyclar AT). After filtering through cheesecloth, the homogenate was centrifuged at 45,000*g* for 20 minute at 4 C and the protein concentration estimated. Cinnamoyl-CoA reductase activity was determined by following the oxidation of cinnamaladehyde at 366 nm in a reaction coupled to the reduction of $NADP^+$. Aliquots of the clarified homogenate were added to the reaction mixture containing 0.1 μM cinnamaldehyde, 0.35 μM coenzyme A, 0.25 μM $NADP^+$, 2 mM Tris-Cl, pH 7.8 in a final volume of 1 ml at room temperature. Cinnamyl alcohol dehydrogenase activity was determined by the oxidation of coniferyl alcohol and cinnamyl alcohol as substrates in a reaction coupled to the reduction of $NADPH + H^+$. Aliquots of the homogenate were added to the reaction mixture containing 1 μM coniferyl or cinnamyl alcohol, 2 μM $NADP^+$, 1 mM Tris-Cl, pH 8.8, and enzyme activity was determined by monitoring the increase in absorbance at 390 nm. All experiments were replicated at least six times, and results are reported as mean ± SEM.

Glyceollin: Soybean plants were harvested and the roots quickly rinsed in sterile distilled water. The roots were frozen in liquid nitrogen, ground in boiling ethanol, and stirred for 30 minutes. The slurry was centrifuged at 17,500*g* for 10 minutes. The aqueous phase was recovered and the residue homogenized a second time in hot ethanol. The ethanolic phases were pooled and concentrated in a flash evaporator.

The extract was dissolved in ethylacetate (1.5 volume/g original wet weight), filtered over $MgSO_4$, and concentrated *in vacuo* at 45 C. The residue was dissolved in 1 ml $CHCl_3$; the absorbance spectrum was recorded between 230 and 400 nm. An aliquot of the extract was spotted onto 250-μm-particle-size silica gel, thin-layer chromatography plates along with an aliquot of a purified glyceollin standard. The plates were developed in $CHCl_3$:H_3COCH_3:NH_4OH (50:50:1). Glyceollins, identified by comparisons to the standards, were scrapped from the plate, eluted from the silica gel with $CHCl_3$, and quantitated by absorbance at 284 nm (13–16).

Results

Enzymes of the central phenylpropanoid pathway: There was no significant difference in the constitutive level of PAL mRNA transcription in uninfected, undamaged, Davis seedlings as compared with Centennial. There was no change in PAL mRNA transcription through 3 days after infection (not shown); however, 4 days after infection significant changes in the resistant seedlings were apparent. Thus, infection of resistant Centennial seedlings with *M. incognita* induced a significant increase in PAL mRNA transcription (Fig. 2A), and a decrease ($P \leq 0.05$) in PAL transcription was seen in infected Davis seedlings. Wounding did not affect transcription of genes encoding PAL in either Davis or Centennial seedlings (Fig. 2A).

Transcription of PAL mRNA in uninfected Hartwig was greater than in uninoculated Essex or Forrest (Fig. 2B). The level of this transcription was nearly equal

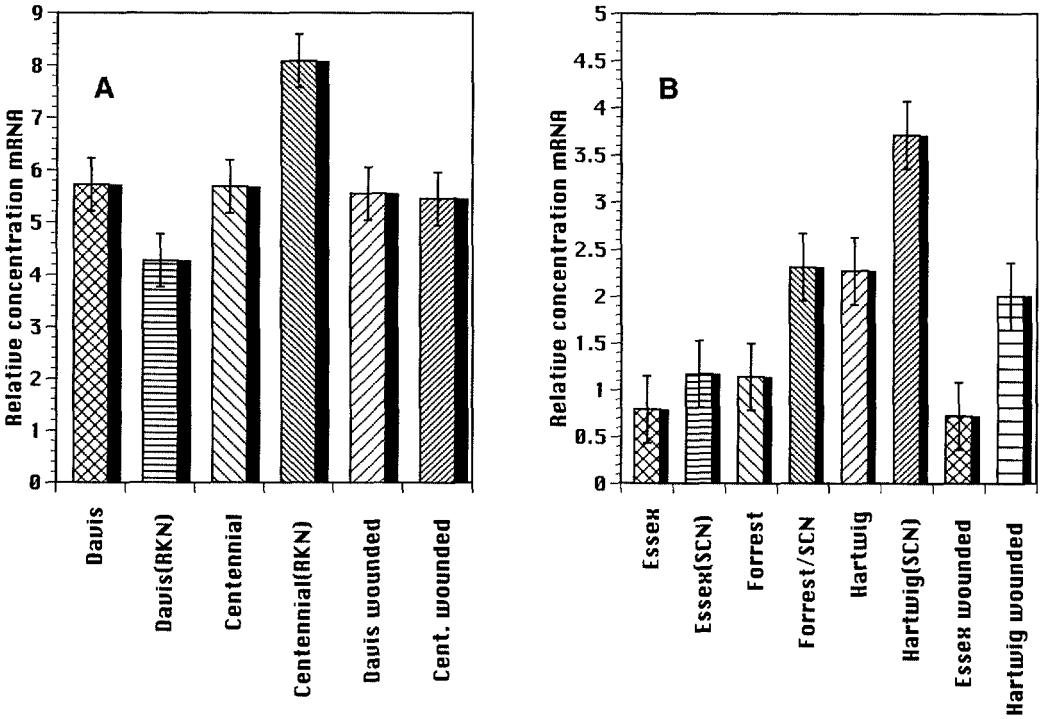

FIG. 2. A. Phenylalanine ammonia lyase mRNA transcription in root tissue of Davis soybean susceptible to *Meloidogyne incognita* race 3 and Centennial soybean resistant to this nematode. The mRNA concentration is the mean of three replicates reported as a relative concentration based on comparison to a densitometric standard. Soybean seedlings were infected in the two-leaf stage with J2 of *Meloidogyne incognita* and analyzed 4 days later. B. Transcription of PAL mRNA in root tissue from resistant (Hartwig and Forrest) and susceptible (Essex) cultivars of soybean infected with *Heterodera glycines* race 3. Plants were infected with J2 of *H. glycines* in the two-leaf stage and analyzed 4 days later. The mRNA concentration is reported as a relative value based on a densitometric standard and is the mean of three replicates.

in Essex and Forrest. When these soybean cultivars were infected with SCN, there was no change in PAL mRNA transcription until 4 days after inoculation, at which time differences ($P \leq 0.05$) occurred depending on the resistance characters of the soybean cultivars. Beginning 4 days after infection, PAL transcription in susceptible Essex seedlings did not differ from that in infected Essex seedlings; however, PAL mRNA transcription increased two-fold above constitutive levels in both Forrest and Hartwig in response to SCN (Fig 2B). Transcription of this mRNA was greatest in Hartwig both before and after infection. Again, wounding had no effect on transcription of PAL mRNA in root tissue from either Hartwig or Essex seedlings.

Induction of transcription of PAL mRNA by *M. incognita* or SCN infection of resistant soybean, but not susceptible soybean, cultivars was accompanied by increased PAL activity (Fig. 3). The activity of PAL was not different in uninfected Davis and Centennial seedlings. Four days after infection with *M. incognita,* PAL activity decreased in Davis seedlings and increased ($P \leq 0.05$) in Centennial seedlings (Fig. 3A). Phenylalanine ammonia lyase activity in uninfected Essex and Forrest seedlings was not different, but the activity of this enzyme was elevated ($P \leq 0.05$) in uninfected Hartwig seedlings (Fig. 3B). Four days after infection with SCN race 3, PAL activity decreased in Essex seedlings but increased ($P \leq 0.05$) in both Forrest and

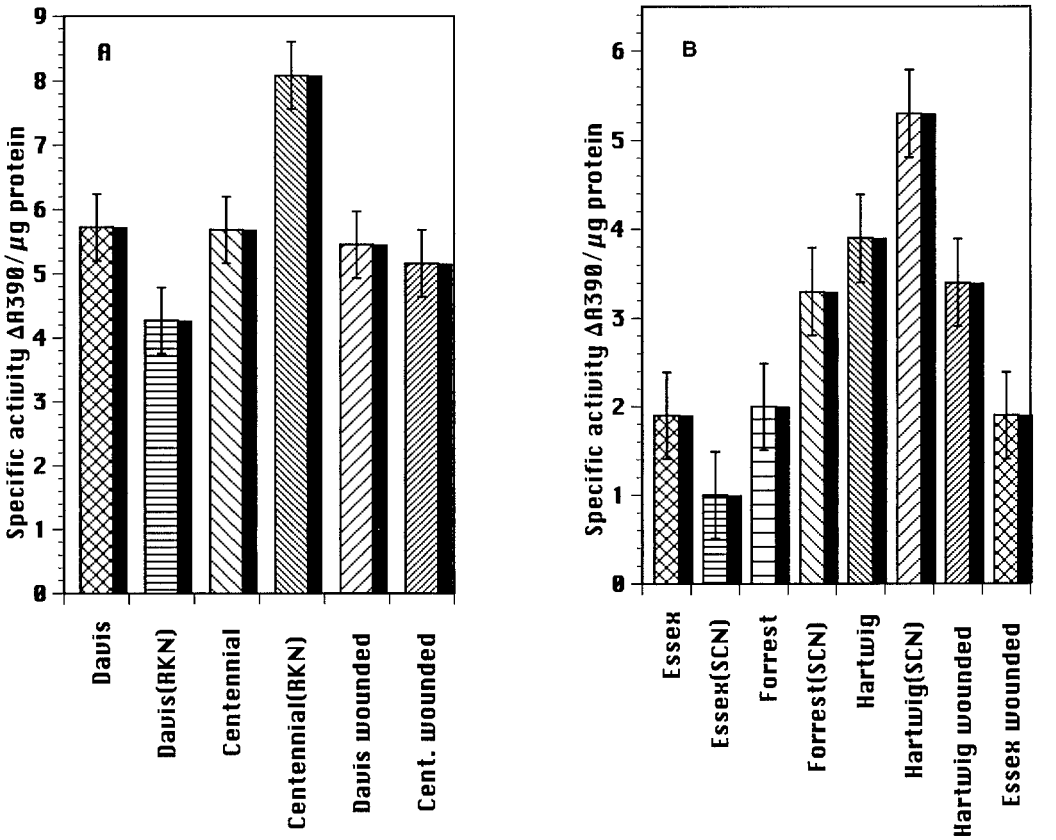

FIG. 3. A. Activity of phenylalanine ammonia lyase in resistant (Centennial) and susceptible (Davis) soybean seedlings 4 days after infection with J2 of *Meloidogyne incognita* race 3. Activity is reported as the μ moles of transcinnamic acid produced per μg of protein in a 1-hour assay. Values are the means of six determinations ± SEM. Soybean was infected at the two-leaf stage. B. Activity of phenylalanine ammonia lyase in resistant (Hartwig and Forrest) and susceptible (Essex) soybean seedlings 4 days after infection with J2 of *Heterodera glycines* race 3. Activity is reported as the μ moles of transcinnamic acid produced per μg of protein in a 1-hour assay. Values are the means of six determinations ± SEM. Soybean was infected at the two-leaf stage.

Hartwig seedlings. Greatest activity was in SCN-infected Hartwig (Fig. 3B). Mechanical wounding did not affect PAL activity in any of the seedlings, i.e., it induced neither an increase or decrease.

The transcinnamic acid produced in the PAL-catalyzed reaction is hydroxylated to 4-coumarate. This molecule then is esterified with coenzyme A in a reaction catalyzed by 4-coumarate CoA ligase (4-CL) to produce 4-coumaryl CoA in the committed step to the phenylpropanoid pathway (Fig. 1). Transcription of 4-CL mRNA was slightly, but not significantly, greater in uninfected resistant Centennial seedlings as compared with uninfected susceptible Davis seedlings (Fig. 4A). Infection of Davis seedlings with *M. incognita* resulted in an apparent doubling of transcription of 4-CL mRNA, but this increase was not statistically significant.

Similar results were seen when the effect of SCN race 3 infection of resistant and susceptible soybean on 4-CL mRNA transcription was compared. Thus, constitutive levels of 4-CL transcription were higher in uninfected, resistant Hartwig seedlings compared with uninfected Essex seedlings (Fig 4B). Soybean cyst nematode infection induced transcription of 4-CL in both the resistant Hartwig and the susceptible Essex seedlings (Fig. 4B). Mechanical wounding had no effect on 4-CL mRNA transcription in any seedlings tested.

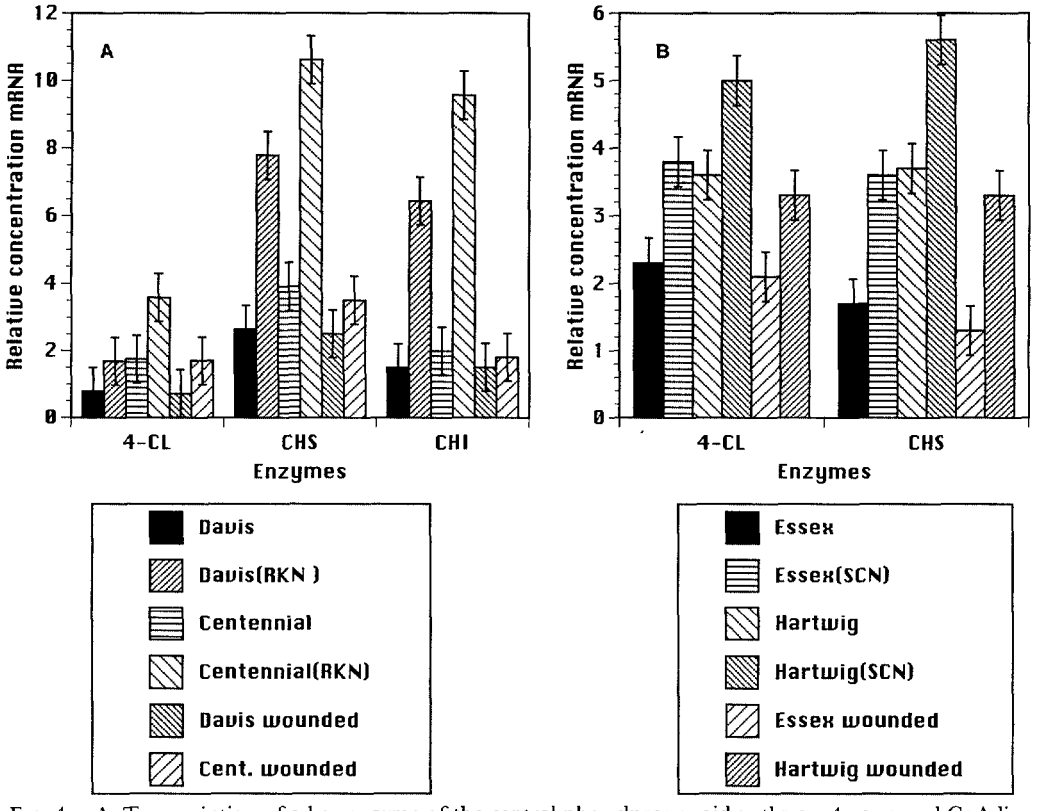

FIG. 4. A. Transcription of a key enzyme of the central phenylpropanoid pathway, 4-coumaryl CoA ligase (4-CL), and of enzymes of the branch of this pathway leading toward biosynthesis of glyceollin, chalcone synthase (CHS), and chalcone isomerase (CHI), in resistant (Centennial) and susceptible (Davis) soybean seedlings 4 days after infection with J2 of *Meloidogyne incognita* race 3. The relative concentration of mRNA is the mean ± SEM of three replicates and is based on comparison to a densitometric standard. B. Transcription of a key enzyme of the central phenylpropanoid pathway, 4-coumaryl CoA ligase (4-CL), and of an enzyme of the branch of this pathway leading toward biosynthesis of glyceollin, chalcone synthase (CHS), in resistant (Hartwig) and susceptible (Essex) soybean seedlings 4 days after infection with J2 of *Heterodera glycines* race 3. The relative concentration of mRNA is the mean ± SEM of three different assays and is based on comparison to a densitometric standard.

Enzymes of branches of the phenylpropanoid pathway: Chalcone synthase (CHS) and chalcone isomerase (CHI) catalyze two major reactions leading to the biosynthesis of isoflavonoids, such as the biologically active glyceollin in soybean. Transcription of CHS and CHI was comparable in uninfected Centennial and Davis soybean cultivars. Increased transcription of CHS and CHI was induced in both cultivars of soybean by infection with *M. incognita* race 3. Although nematode-induced transcription of CHS and CHI was greatest in Centennial, nematode infection of Davis seedlings induced a significant increase in transcription of mRNA encoding both enzymes (Fig. 4A). Transcription of CHS was greater in uninfected Hartwig than in uninfected Essex. Soybean-cyst nematode race 3 infection of both Essex and Hartwig seedlings induced increased transcription of CHS (Fig. 4B). Mechanical wounding did not affect transcription of either of these enzymes in any of the seedling cultivars assayed.

The increase in transcription of CHS and CHI mRNA in *M. incognita*-infected Centennial seedlings was coincident with increased glyceollin synthesis 4 to 7 days after nematode infection (Fig. 5). Glyceollin synthesis did not increase in Davis seedlings 4 or 7 days after infection with *M. incognita* race 3.

Enzymes of the pathway to lignin biosynthesis: Activities of two key enzymes, cinnamyl alcohol dehydrogenase (CAD) and cinnamyl CoA:NADPH oxidoreductase (CoCAR), leading to synthesis of lignin were not significantly different in susceptible Davis and resistant Centennial seedlings. The activity of these enzymes was not induced by infection of either soybean cultivars with *M. incognita* race 3 (Fig. 6).

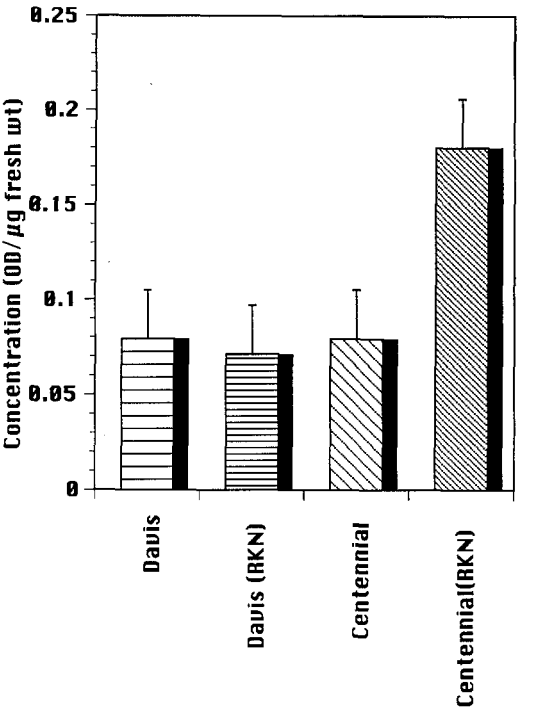

FIG. 5. Glyceollin synthesis in resistant (Centennial) and susceptible (Davis) soybean seedlings infected with J2 of *Meloidogyne incognita* race 3. Seedlings were infected at the two-leaf stage and assayed for glyceollin I concentration 4 and 7 days later. Glyceollin was extracted as described by Huang and Barker (13) and separated by thin-layer chromatography. The concentration was estimated in material recovered from thin-layer chromatograms, using a glyceollin standard (13) chromatographed in parallel.

DISCUSSION

Increased activity of enzymes at the initiation of the phenylpropanoid pathway (PAL, cinnamate-4 hydroxylase, and 4-coumaryl CoA ligase), of enzymes leading to flavonoid and isoflavonoid biosynthesis (chalcone synthase and chalcone isomerase), and of enzymes leading to synthesis of specific phytoalexins has been reported in some legumes following treatment with elicitors from fungal or yeast cell walls (9). Similar changes are induced by SCN and *M. incognita* in soybean cultivars resistant to these nematodes. That is, major enzymes of the central phenylpropanoid pathway and of the branch leading to glyceollin synthesis were transcribed at higher levels in resistant soybean cultivars compared to susceptible cultivars. Increased PAL mRNA transcription, after nematode infection of resistant cultivars, resulted in increased enzyme activity, indicating that the transcribed products were translated into active enzymes in these plants. Constitutive mRNA transcription and (or) activity of some enzymes of the phenylpropanoid pathway were greater in

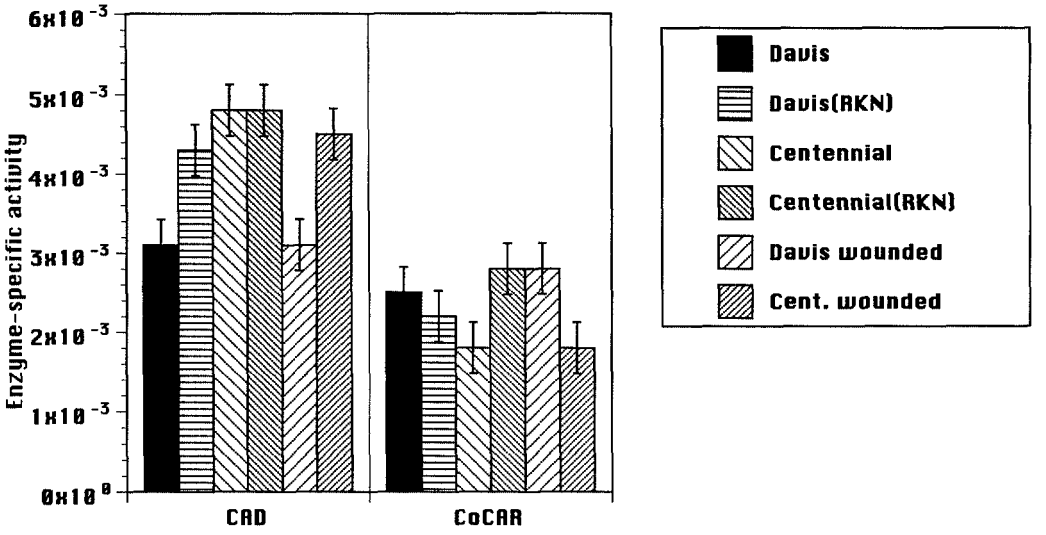

FIG. 6. Activity of cinnamyl alcohol dehydrogenase (CAD) and cinnomyl CoA reductase (CoCAR) in resistant (Centennial) and susceptible (Davis) soybean seedlings 4 days after infection with J2 of *Meloidogyne incognita* race 3. CAD activity is reported as the rate of change in absorbance at 366 nm per μg protein per minute as NADP is reduced to NADPH in reaction coupled to the oxidation of cinnamyl alcohol to cinnamylaldehyde. CoCAR activity is reported as the rate of change in absorbance at 340 nm per μg protein per minute as NADPH is oxidized to NADP in a reaction coupled to the reduction of cinnamyl CoA. The results are the mean ± SEM of six determinations.

the resistant cultivars, suggesting preparation of the plant to respond to pathogen invasion. The changes in response to nematode infection occurred coincident with the reported timing of the development of nematode feeding sites and cellular changes at the feeding site associated with the resistance response in resistant cultivars (12). Changes in transcription of PAL, 4-CL, CHS, and CHI were greatest in resistant cultivars: however, CHS, CHI, and perhaps 4-CL increased in susceptible cultivars after nematode infection. Transcription of PAL decreased after infection of Davis seedlings with *M. incognita* and increased slightly after infection of Essex seedlings with SCN, but PAL activity declined in both susceptible cultivars following nematode infection. This decrease is comparable to that seen in susceptible tomato and carrot roots infected with *Meloidogyne* spp. (3,4). These observations lead to the reasonable suggestion that nematode infection induces not only up-regulation of transcription of specific enzymes, but also down-regulation of transcriptional, translational, or catalytic events. This might occur through elicitation of molecules that function either to modulate gene transcription or to regulate catalytic activity of specific enzymes. Several isoforms of PAL have been implicated in resistance to fungal elicitors (6,9). For example, in resistant alfalfa cultivars, the induced PAL isoform had a high K_m for phenylalanine, indicating substrate-level regulation (9). Although we did not evaluate induction of isoforms of PAL in soybean by SCN or *M. incognita,* it might be suggested that genetic differences between resistant and susceptible cultivars are reflected in differential induction of the members of the multigene family encoding PAL in soybean (7).

Nematode infection caused a significant induction of the transcription of some genes encoding enzymes of the branch of the phenylpropanoid pathway leading toward isoflavonoid biosynthesis. Specifically, transcription of chalcone synthase and chalcone isomerase transcription were enhanced in both susceptible and resistant cultivars following nematode infection. Since glyceollin concentration increases

only in resistant cultivars following nematode infection (13–16), our observations suggest control of this branch of the pathway may be at translation of mRNA to a functional protein, post-translational modification of the protein, turnover of the mRNA, or catalytic mechanism of the enzyme. Increased transcription of CHS and CHI in infected susceptible cultivars of soybean suggests increased availability of these enzymes for synthesis of glyceollin. That increased glyceollin synthesis does not occur and that PAL activity is decreased in infected susceptible cultivars suggest that insufficient transcinnamic acid is produced as a substrate for the initial and committed reaction of this pathway toward protection against the nematodes. That is, the lack of a precursor at the initiation of the pathway may lead to a bottle neck for subsequent product synthesis. The ability of susceptible soybean cultivars to produce sufficient glyceollin as a protection against *M. incognita* and SCN might be delayed until after the nematode has established and mounted a protective barrier against the effect for the phytoalexin. This result might explain the observations of Kaplan et al. (15) and Huang and Barker (13) that glyceollin accumulates immediately after infection of resistant soybean with *M. incognita* or SCN and much later in susceptible cultivars. Regardless of the mechanism of regulation, it is apparent that there is some degree of specificity to the response induced by nematode infection of soybean since there was no change in key enzymes associated with the branch of the phenylpropanoid pathway leading toward lignin synthesis. Implied, but not proven, is that the host may respond specifically to the elicitor from the nematode immediately after infection, and that this response may be most effective in resistant cultivars. If this is true, then it suggests that resistance genes encode a product(s) that functions to regulate transcription of genes key to the enzymatic pathway involved with soybean resistance to *M. incognita* and SCN (13–16), or they may encode elicitor receptors or signal-transducing molecules. Results from studies on *M. incognita* infection of soybean or tomato strongly suggest that the early events of recognition are critical to establishment or rejection of the nematode and that these events begin immediately after infection and reach their maximum expression within 72 to 96 hours (5). Recently, Levin et al. (20) demonstrated an oxidative burst within 2 to 3 hours of elicitation of a hypersensitive response in resistant soybean cultivars infected with *Pseudomonas syringae* pv *glycinea* characterized by a sustained accumulation of H_2O_2. These investigators suggest that this burst drives the cross-linking of cell wall structural proteins to protect against pathogen establishment. They were unable to establish a role for superoxide dismutase (SOD) in this rapid oxidative burst following *P. syringae* pv *glycinea* infection. Similarly, Vanderspool et al. (28) were unable to demonstrate a role of SOD in the resistance of soybean to nematode infection; however, their results do suggest that SOD may have some role in the plant-nematode interaction in both susceptible and resistant cultivars. The involvement of peroxides, SOD, and peroxidases in the interaction of *M. incognita* or SCN with resistant and susceptible soybean cultivars needs to be investigated further in light of these recent findings.

On the basis of the observations reported, it appears that soybean cultivars resistant to both SCN and *M. incognita* respond to nematode infection by induction of transcription of enzymes of pathways leading to production of chemical protection against the pathogen. There are many possible mechanisms by which soybean increases concentrations or activity of enzymes needed to provide defense against nematode infection, including the possibility of activation of genes encoding specific DNA-binding proteins to induce transcription of key genes or of the presence of a larger number of copies of key genes in resistance germplasm. The exact mechanism is still unknown, and its induction and relationship to the genetics of resistance need further investigation.

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