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Differential Expression of Phenylalanine Ammonia-Lyase and Chalcone Synthase during Soybean Nodule Development

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We have used conserved and nonconserved regions of cDNA clones for phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) isolated from a soybean-nodule cDNA library to monitor the expression of members of the two gene families during the early stages of the soybean-*Bradyrhizobium japonicum* symbiosis. Our results demonstrate that subsets of the PAL and CHS gene families are specifically induced in soybean roots after infection with *B. japonicum*. Furthermore, by analyzing a supernodulating mutant line of soybean that differs from the wild-type parent in the number of successful infections, we show that the induction of PAL and CHS is related to postinfection events. Nodulated roots formed by a Nod$^+$ Fix$^-$ strain of *B. japonicum*, resembling a pathogenic association, display induction of another distinct set of PAL and CHS genes. Our results suggest that the symbiosis-specific PAL and CHS genes in soybean are not induced by stress or pathogen interaction.

INTRODUCTION

Phenylpropanoid compounds synthesized by plants have various roles in plant growth and development. Examples of these compounds and their functions are the lignins for structural integrity and barriers, flavonoid pigments for color and UV protection, and phytoalexins as antibiotics (Hahlbrock and Scheel, 1989). Additionally, phenylpropanoid compounds act as signals in plant-microbe interactions.

Flavonoids are the first known signals involved in the establishment of the *Rhizobium*-legume symbiosis. During the initial interaction between *Rhizobium* and legumes, host-derived flavonoid compounds interact with the NodD protein to activate transcription of other *nod* genes (Peters et al., 1986; Redmond et al., 1986; Kosslak et al., 1987; Peters and Long, 1988). However, some flavonoid compounds inhibit *nod* gene transcription (Firmin et al., 1986; Peters and Long, 1988). Furthermore, after inoculation with *Rhizobium leguminosarum*, the roots of *Vicia sativa* produce additional *nod* gene-inducing flavonoids (Van Brussel et al., 1990).

Plant-pathogen interactions result in the induction of host defense responses that include the production of phenylpropanoid compounds (Dixon and Lamb, 1990). Various stages of nodule development resemble plant-pathogen interactions (Vance, 1983; Djordjevic et al., 1987), yet the host does not produce a host defense response against the symbiont.

Two key enzymes in the biosynthesis of phenylpropanoid compounds are phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) (Smith and Banks, 1986). The first reaction in the pathway is the deamination of phenylalanine to cinnamic acid by PAL. Further along in the pathway, CHS condenses three molecules of malonyl CoA with cinnamyl CoA to produce chalcone. This condensation represents a major branch point in the pathway for the specific production of flavonoids. In many plant species, PAL and CHS are encoded by multigene families (Koes et al., 1987; Niesbach-Klosgen et al., 1987; Ryder et al., 1987; Cramer et al., 1989; Hahlbrock and Scheel, 1989; Wingender et al., 1989). Individual members of the two gene families are differentially regulated and utilize different signals for induction (Kreuzaler et al., 1983; Bell et al., 1986; Ryder et al., 1987; Koes et al., 1989; Liang et al., 1989; Harker et al., 1990).

The work presented in this paper focuses on the expression of members of the PAL and CHS gene families during the early stages of soybean root-*Bradyrhizobium japonicum* symbiosis and during the developmental stages leading to effective or ineffective symbiosis. Using conserved and nonconserved regions of cDNA clones for PAL and CHS isolated from a nodule cDNA library, we demonstrate that subsets of both gene families are induced after specific infection by *B. japonicum*. Furthermore, the subset induced early in symbiosis is different from gene members induced as a host defense or stress response during the later stages of ineffective symbiosis.

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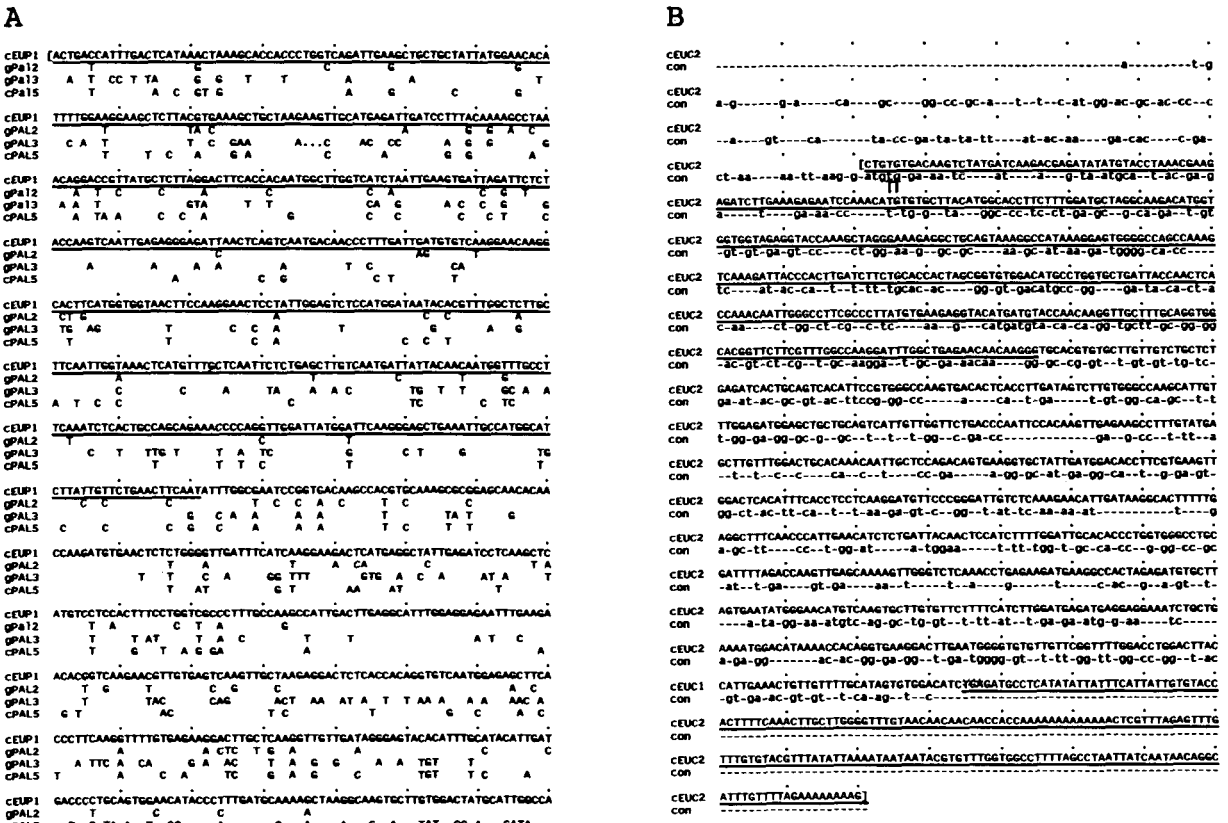

Figure 1. Nucleotide Sequence of Soybean cDNA Clones for PAL and CHS and Alignment to Corresponding Sequences.

Blank spaces indicate identical bases among the sequences; nonidentical bases are noted. A period (·) within the sequence indicates sequence gaps to maximize alignment. The beginnings and ends of sequences are bracketed. Underlined portions of sequences are restriction fragments used as probes in further experiments. Start and stop codons are shaded.

(A) Alignment of the entire PAL cDNA sequence isolated from soybean (cEUP1), partial second exon sequences of French bean genomic clones (gPAL2 and gPAL3) (Cramer et al., 1989), and partial cDNA sequence from French bean (cPAL5) (Edwards et al., 1985).

(B) Alignment of CHS cDNA sequence isolated from soybean (cEUC2) and a CHS consensus sequence (con) (Niesbach-Klosgen et al., 1987). Dashes in the consensus sequence are nonconserved nucleotides.

RESULTS

Conserved and Nonconserved Regions Identified in Soybean PAL and CHS cDNAs Isolated from a Nodule cDNA Library

A cDNA library to poly(A) RNA isolated from nodules (cv Prize) 14 days postinoculation (DPI) was screened with the French bean cDNA inserts of PAL (Edwards et al., 1985) and CHS (Ryder et al., 1984). Three clones each for PAL and CHS were isolated from 4×10^5 plaques. Based on restriction enzyme digestion patterns, the three clones in each case were found to be identical.

The cDNA inserts of the isolated clones were sequenced and compared with the same genes from other plants. The sequence alignment shown in Figure 1 revealed conserved

and nonconserved regions. Alignment with the three PAL genes from French bean (Edwards et al., 1985; Cramer et al., 1989) showed that the 1427-bp soybean PAL cDNA contained only the second exon (Figure 1A). Although the 5′ translated regions of the second exons were conserved among the genes, the 3′ untranslated regions were nonconserved. The 1188-bp soybean CHS cDNA was aligned with the French bean CHS genes (alignment not shown) and a consensus CHS sequence (Niesbach-Klosgen et al., 1987). According to the alignment (Figure 1B), the soybean CHS cDNA was found to be incomplete and contained only 4 bases of the first exon and the entire second exon. Again, as in the PAL genes, the 5′ translated regions were conserved among the genes, whereas the 3′ untranslated regions were nonconserved.

The 3′ Nonconserved Regions Hybridize to Subsets of Genes Identified by the 5′ Conserved Regions

To determine whether the 3′ nonconserved regions from the nodule-derived cDNA clones are gene specific, DNA gel blots of genomic DNA were hybridized with fragments from the 5′ conserved and 3′ nonconserved regions. The restriction enzyme fragments used as probes are underlined in Figure 1. The 5′ PAL and 5′ CHS probes represented conserved translated regions, whereas the 3′ CHS probe was from the nonconserved untranslated region. The 3′ PAL probe, besides containing the untranslated region, contained 35 bp of the 3′ translated region. Genomic DNA was digested with EcoRI and HindIII because these sites do not occur within the nodule-derived cDNA clones. The four DNA gel blots were hybridized and washed using the same conditions. Figure 2 shows that the 5′ PAL probe hybridized to three HindIII fragments of 8.1 kb, 3.6 kb, and 0.74 kb and six EcoRI fragments of 23.0 kb, 15.0 kb, 9.4 kb, 2.7 kb, 1.7 kb, and 1.6 kb, whereas the 3′ PAL probe hybridized only to the 8.1-kb and 3.6-kb HindIII fragments and the 9.4-kb and 1.6-kb EcoRI fragments. The 3′ PAL probe displayed a relatively higher level of hybridization with the 1.6-kb EcoRI and the 3.6-kb HindIII fragments. These results suggest that although there are potentially six genes encoding PAL in soybean, the 3′ probe has sequence homology to only two of the genes.

DNA gel blots were also probed with the conserved 5′ translated and nonconserved 3′ untranslated regions of the CHS cDNA clone (Figure 2). Although the conserved 5′ probe of CHS hybridized to eight HindIII fragments of 20.0 kb, 10.0 kb, 7.4 kb, 4.3 kb, 3.9 kb, 3.4 kb, 2.3 kb, and 1.9 kb and three EcoRI fragments of 19.0 kb, 16.0 kb, and 5.4 kb, the nonconserved 3′ probe hybridized only to the 20.0-kb, 10.0-kb, and 3.4-kb HindIII and 19.0-kb and 16.0-kb EcoRI fragments. These results suggest that although there are potentially eight genes encoding CHS, the 3′ CHS probe has sequence homology to only three of the genes.

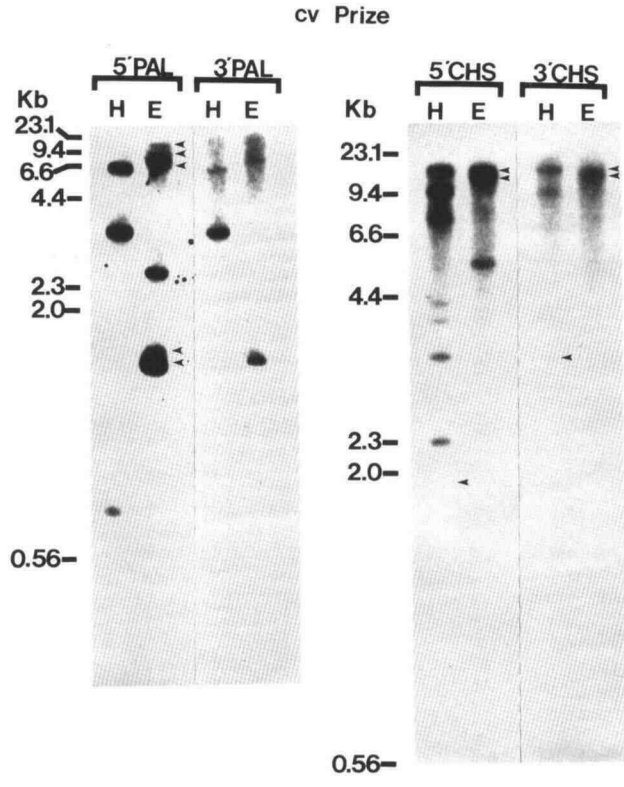

Figure 2. DNA Gel Blot Analysis of Soybean Genomic DNA.

Genomic DNA (10 μg) isolated from leaf tissue (cv Prize) was digested with HindIII (H) or EcoRI (E) and fractionated on a 1.0% agarose gel. Replica blots on nitrocellulose were hybridized to different probes as indicated. DNA restriction enzyme fragments used as probes are underlined in Figure 1. 5′ PAL and 5′ CHS are the 5′ translated conserved regions of PAL and CHS genes, whereas the 3′ PAL and 3′ CHS are the 3′ nonconserved regions of PAL and CHS. Positions of the molecular weight markers are indicated on the left. Arrows indicate distinct bands present in the autoradiograph but not resolved in the photograph.

PAL and CHS Probably Are not Classically Defined Nodulins

Because the clones for CHS and PAL were isolated from a cDNA library to nodule poly(A) RNA, the clones were checked to determine whether they represented nodulins. Nodulins are defined traditionally as proteins that are synthesized in nodules but not in roots (Van Kammen, 1984). RNA gel blots of poly(A) RNA isolated from roots and nodules were probed with the 5′ conserved and 3′ nonconserved regions for both PAL and CHS genes. Figure 3

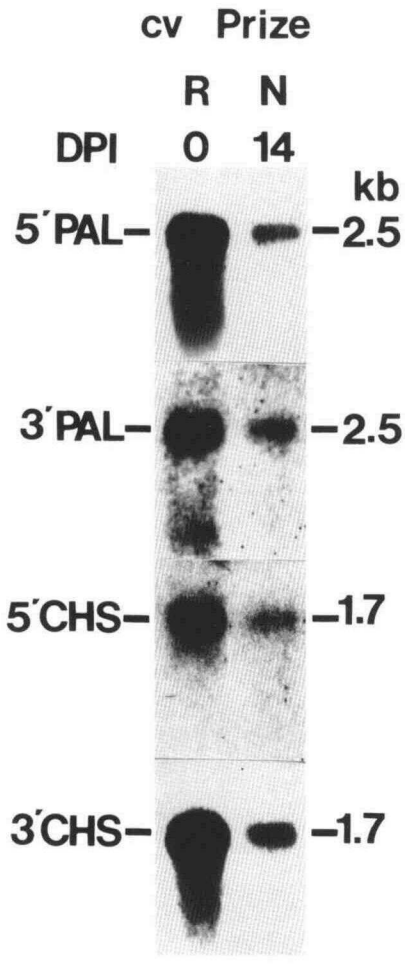

Figure 3. RNA Gel Blot Analysis of Polysomal Poly(A) RNA Isolated from Roots and Nodules.

Polysomal poly(A) RNA (2.5 μg) from uninoculated roots (R) (0 DPI) and nodules (N) collected 14 DPI of cv Prize with USDA 110 was subjected to RNA gel blot analysis using the 5′ and 3′ probes of PAL and CHS as indicated. Molecular weights of transcripts in kilobases were determined by comparing with coelectrophoresed RNA markers.

shows that both gene probes had a higher hybridization level to root RNA than to nodule RNA. The RNA transcript size detected for PAL was 2.5 kb, whereas the CHS transcript size was 1.7 kb. According to the hybridization pattern, the genes identified by the 3′ probes are expressed in both roots and nodules and, thus, do not represent nodulins. However, because the 3′ probes hybridize to a subset of genomic fragments, we cannot rule out the possibility that a unique member is expressed in the nodules.

The 3′ Nonconserved Regions Identify Symbiosis-Specific PAL and CHS Gene Subsets

Because flavonoid compounds play a crucial role during the early stages of nodule development (Firmin et al., 1986; Peters et al., 1986; Redmond et al., 1986; Kosslak et al., 1987; Peters and Long, 1988), the expression of PAL and CHS genes was monitored in uninoculated roots and roots inoculated with *B. japonicum* (USDA 110). To facilitate analysis of changes in gene expression associated with infection, we used the supernodulating mutant *nts*382 (Carroll et al., 1985), which has 40% more successful infections than the wild-type parent cv Bragg (Gresshoff et al., 1988). Figure 4 shows duplicate RNA gel blots of poly(A) RNA isolated from uninoculated and inoculated roots of *nts*382 hybridized to the 5′ conserved and 3′ nonconserved probes for both PAL and CHS. Because the probes differed in size and exposure times of the autoradiograms for the 5′ and 3′ probes were not identical, the absolute hybridization signals between the 5′ and 3′ probes cannot be compared.

The 5′ probes for both PAL and CHS showed a slight increase in the level of transcripts in uninoculated roots at 4 days (Figure 4). The 3′ PAL probe revealed a drop in transcript accumulation at 1 day and 2 days, followed by an increase at 4 days, whereas the 3′ CHS probe showed an increase in transcript accumulation at 1 day and 2 days and a drop at 4 days. The differences in the hybridization patterns between the 3′ PAL and CHS probes cannot be attributed to errors in the RNA loads because the same filter was hybridized with the two probes.

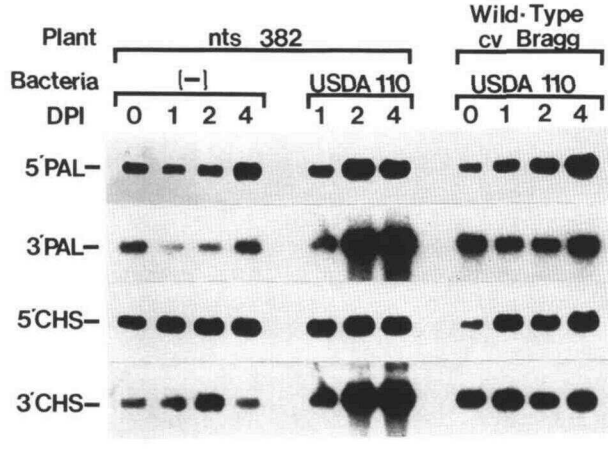

Figure 4. RNA Gel Blot Analysis of Total Poly(A) RNA Isolated from Developing and Inoculated Roots.

Total poly(A) RNA (2.5 μg) isolated from *nts*382 and wild-type cv Bragg uninoculated roots or roots at different days postinoculation with USDA 110 were subjected to RNA gel blot analysis using the 5′ and 3′ probes of PAL and CHS as indicated.

With inoculated *nts*382, the 5′ PAL and 5′ CHS probes detected an increase in the transcript level for the first 2 DPI and a slight drop at 4 DPI. The 3′ probes for both PAL and CHS showed a dramatic increase in the transcript level at 2 DPI and 4 DPI. The relative levels of hybridization signals between uninoculated and inoculated roots, as assessed by the conserved and nonconserved probes, were not the same. Proportional increases in transcripts detected by the 5′ probes and the 3′ probes after inoculation could not be detected. This suggests that expression of some members of the PAL and CHS gene families decrease, whereas gene members identified by the 3′ probes increase in expression after infection with *B. japonicum*.

To determine whether the increased expression of specific subsets of the PAL and CHS gene families can be attributed to the initial interaction between the host and the symbiont or to a postinfection stage, the expression of PAL and CHS in *nts*382 was compared with the wild-type parent cv Bragg. Gresshoff et al. (1988) showed that cv Bragg differed from *nts*382 in the number of nodules formed. Although the number of initial root infections was the same in *nts*382 and cv Bragg, the number of aborted infections was fewer in *nts*382 (Gresshoff et al., 1988). The result was a 10-fold increase in the number of nodules formed in *nts*382 (Sutherland et al., 1990). Mathews et al. (1989) could not detect differences in the *nod* gene inducing activity between roots of *nts*382 and cv Bragg before inoculation. However, our results on the analysis of PAL and CHS gene expression in the uninoculated roots of *nts*382 and cv Bragg revealed differences in activity.

Figure 4 shows that the 5′ PAL and CHS probes displayed a lower level of hybridization to root RNA from cv Bragg compared with *nts*382 before inoculation, whereas the 3′ probes had a higher level of hybridization to *nts*382 RNA (0 DPI). At the postinfection stages, cv Bragg transcripts hybridizing to the 5′ and 3′ PAL probes gradually increased. However, 5′ CHS-specific transcripts were higher at 4 DPI, whereas the transcripts detected by the 3′ CHS probe increased starting at 1 DPI. Comparison of the hybridization patterns for the 5′ PAL and CHS probes between cv Bragg and *nts*382 after inoculation revealed a higher level of hybridization to RNA from cv Bragg than to *nts*382 at 4 DPI. However, *nts*382 had a much higher level of hybridization than cv Bragg to the 3′ probes at 2 DPI and 4 DPI. Taken together, these results suggest that the gene members represented by the 3′ nonconserved regions are induced in both cv Bragg and *nts*382 after infection. However, the transcript levels were higher in *nts*382, suggesting that the degree of induction is related to the number of successful infections.

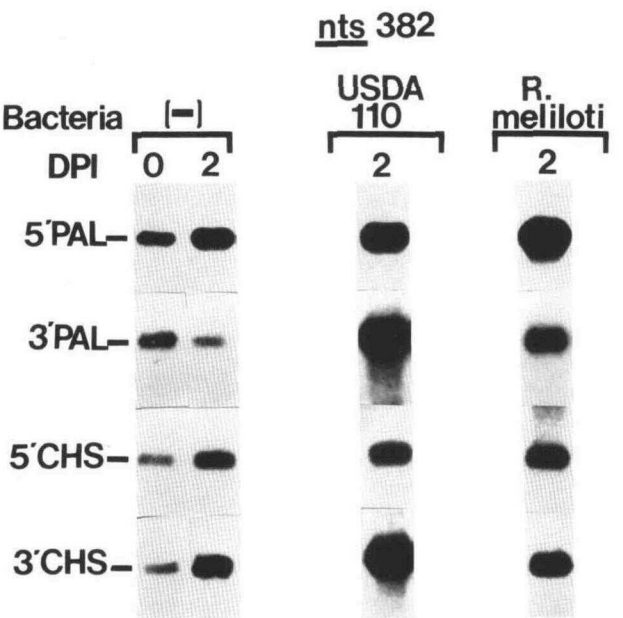

Figure 5. RNA Gel Blot Analysis of Total Poly(A) RNA Isolated from Roots Inoculated with *B. japonicum* Strain USDA 110 or *R. meliloti*.

Total poly(A) RNA (2.5 μg) isolated from *nts*382 uninoculated roots and roots 2 DPI with USDA 110 or *R. meliloti* was subjected to gel blot analysis using the 5′ and 3′ probes of PAL and CHS as indicated.

Induction of the Symbiosis-Specific Gene Subsets Is Specific to Infection with *B. japonicum*

To determine whether the increase in the abundance of PAL and CHS transcripts was specific to inoculation with *B. japonicum*, gene expression was analyzed in roots of *nts*382 inoculated with *Rhizobium meliloti*. Poly(A) RNA isolated from roots at 2 DPI with *R. meliloti* was hybridized with the conserved and nonconserved probes. Figure 5 shows that the 5′ PAL probe hybridized more intensely to transcripts from roots at 2 DPI with *R. meliloti* than with *B. japonicum* (USDA 110). The 3′ PAL probe showed a higher level of hybridization in roots inoculated with *B. japonicum*. Transcripts detected by both 5′ and 3′ PAL probes were more abundant in inoculated roots than in uninoculated roots. The 5′ CHS probe revealed little difference in the level of transcripts between roots inoculated with *B. japonicum* and *R. meliloti*, but their levels were higher than uninoculated roots. Hybridization of the 3′ CHS probe to RNA from roots inoculated with *R. meliloti* did not increase, but a higher level of hybridization was detected in roots inoculated with *B. japonicum*.

Different Members of the PAL and CHS Gene Families Are Expressed in Fix⁻ Associations

Later stages of nodule development formed by Fix⁻ strains of *B. japonicum* resemble pathogenic rather than symbiotic

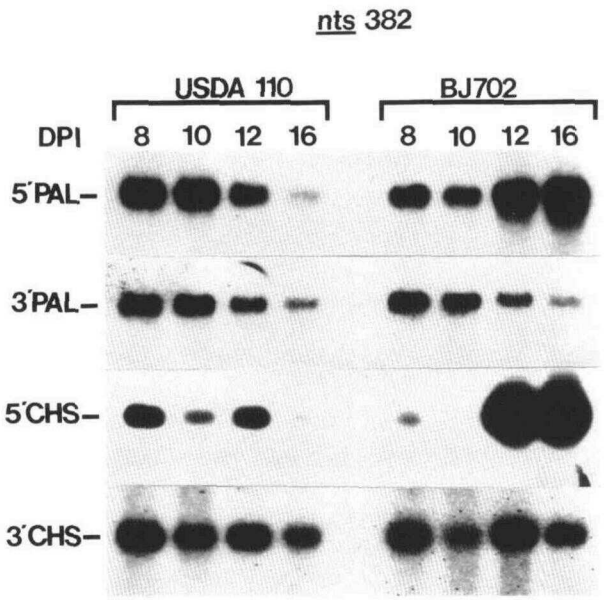

Figure 6. RNA Gel Blot Analysis of Total Poly(A) RNA Isolated from Nodulated Roots during Later Stages of Symbiotic Development with Fix$^+$ and Fix$^-$ *B. japonicum* Strains.

Total poly(A) RNA (2.5 μg) isolated from *nts*382 nodulated root sections was subjected to RNA gel blot analysis using the 5′ and 3′ probes of PAL and CHS. Samples were taken at different days postinoculation with Fix$^+$ (USDA 110) and Fix$^-$ (BJ702) *B. japonicum*.

associations because the symbiont, by not fixing nitrogen, fails to contribute to symbiosis. To investigate whether ineffective symbiosis leads to the induction of PAL and CHS genes, *nts*382 roots inoculated with a Fix$^-$ strain, BJ702 (*nif*KD deletion mutant), were analyzed for the expression of PAL and CHS genes during the later stages of nodule development. Figure 6 shows that the 5′ conserved probes for the PAL and CHS genes gradually decreased in hybridization to RNA from nodulated roots between 8 DPI and 16 DPI with the wild-type Nod$^+$ Fix$^+$ strain USDA 110. However, in roots inoculated with the Fix$^-$ strain BJ702, the level of hybridization with the 5′ probes was lower at 8 DPI and 10 DPI than in roots inoculated with USDA 110 but increased dramatically by 12 DPI and 16 DPI. The 3′ nonconserved probes for the PAL and CHS genes decreased in hybridization to roots nodulated with the Fix$^+$ or Fix$^-$ strains. These results indicate that gene members induced early in the postinfection stage differ from members induced in ineffective symbiosis. Similar hybridization patterns using the 5′ and 3′ probes for PAL and CHS were obtained with isolated nodules from a wild-type cultivar formed by Fix$^+$ and Fix$^-$ strains and the supernodulating mutant (data not shown).

DISCUSSION

Although flavonoids are involved in symbiosis and plant-pathogen interactions, the expression of genes for the enzymes involved in flavonoid biosynthesis has not been studied during nodule development. To study PAL and CHS gene expression during the early stages of infection and nodule development, we used a supernodulating mutant of soybean (Carroll et al., 1985). To differentiate between gene members, conserved and nonconserved domains of cDNA clones for PAL and CHS genes were used as probes. Our results demonstrate that subsets of the PAL and CHS gene families induced in soybean after inoculation with effective *B. japonicum* differ from those induced during the later stages of ineffective symbiosis.

Genomic blots of soybean DNA probed with cDNA clones for PAL and CHS revealed that the two enzymes are encoded by multigene families. Based on sequence analysis of the PAL and CHS cDNA clones, a region of both clones was found to be nonconserved. However, the nonconserved regions hybridized with different intensities to more than one genomic band, suggesting that some members share sequence homology in the 3′ region. Because the probe to the nonconserved region of the particular PAL and CHS genes shares homology to multiple gene members, our studies on gene expression could not distinguish between the members in a subset.

Uninoculated roots showed developmental changes in expression of the PAL and CHS genes. Flavonoid exudation is developmentally controlled in legume roots and corresponds to the zone of maximum infection (Redmond et al., 1986; Peters and Long, 1988). Sutherland et al. (1990) found that older roots of soybean have lower *nod* gene-inducing ability than younger roots. Flavonoid compounds secreted by roots also differ qualitatively and quantitatively in the particular types of flavonoids produced (Parniske et al., 1988; Maxwell et al., 1989). The localized sites of flavonoid exudation and developmental differences in *nod* gene-inducing ability could account for the changes in expression of the PAL and CHS genes in uninoculated soybean roots.

Both the supernodulating mutant *nts*382 and the wild-type cv Bragg showed an increase in the level of the transcripts identified by the 3′ nonconserved probes for both PAL and CHS genes between 1 DPI and 4 DPI. However, the increase in the transcript level was more dramatic in *nts*382 than in cv Bragg between 2 DPI and 4 DPI. In view of the fact that *nts*382 differs from cv Bragg in the number of successful infections (Gresshoff et al., 1988), we suggest that the increased expression of the specific PAL and CHS genes is correlated with differences in the ability to establish infection in the two hosts. However, we have not determined whether the increased levels of the symbiosis-specific PAL and CHS transcripts are a cause or a result of the decrease in aborted infections.

Kapulnik et al. (1987) provided evidence for a direct correlation between the amount of flavonoids exuded by alfalfa and the number of nodules formed on their roots. They also showed that direct application of luteolin (flavonoid inducer of *R. meliloti nod* genes) to the rhizosphere of alfalfa resulted in increased nodulation. Thus, it is possible that flavonoid biosynthesis is a component of autoregulation in soybeans.

The increase in the transcripts for PAL and CHS in the roots of *nts*382 between 2 DPI and 4 DPI appears to be accompanied by a decrease in the expression of family members that can be detected by the 5′ conserved probe. These changes in abundance of PAL and CHS transcripts in the inoculated *nts*382 roots could represent a decrease in the synthesis of particular flavonoid antagonists. Legumes have been shown to produce both flavonoid inducers and antagonists of *nod* genes (Firmin et al., 1986; Peters et al., 1986; Redmond et al., 1986; Kosslak et al., 1987; Peters and Long, 1988).

Inoculation of *nts*382 with the heterologous bacterium *R. meliloti* yields a different pattern of gene expression than that after inoculation with *B. japonicum*. The results suggest that inoculation with *R. meliloti* causes a general induction of the PAL and CHS gene families. However, this general induction differs from the very high stimulation of PAL-specific and CHS-specific members after infection with the compatible bacterium *B. japonicum*.

An ineffective association initiated by a Nod^+ Fix^- strain resulted in the induction of gene members for PAL and CHS that differ from those induced early in symbiosis. Host defense responses to fungal pathogens in soybean are accompanied by increased expression of PAL and CHS genes that lead to the synthesis of the phytoalexin glyceollin I (Ebel et al., 1984). Werner et al. (1985) have shown that in some symbiotic associations formed by Nod^+ Fix^- bacteria glyceollin I accumulates in the later stages of nodule development. The Nod^+ Fix^- strain BJ702 (B. Chelm, personal communication) resembles the wild-type strain USDA 110 except for the ability to produce a functional nitrogenase enzyme. The timing of induction (12 DPI) of the PAL and CHS genes in roots nodulated by BJ702 coincides with the timing of steady-state nitrogen fixation in nodules formed by USDA 110. Hence, the dramatic induction of PAL and CHS genes could be a response to nitrogen stress or a defense response to the endoparasite BJ702. The symbiosis-specific genes identified by the 3′ nonconserved probes had an identical pattern of expression in roots nodulated with USDA 110 and BJ702, suggesting that these genes are regulated by events occurring early in symbiosis and are not induced by stress or a pathogen. Conversely, these results suggest that infection with the compatible symbiont does not induce the gene members that are related to host defense.

In most of our analyses, PAL and CHS genes appeared to be coordinately regulated. However, in some instances, differences in the patterns of expression between the two gene families were observed. For example, during the development of uninoculated roots, expression, as monitored by the 3′ PAL probe, was different from the expression pattern obtained with the 3′ CHS probe (Figure 4). The phenylpropanoid pathway has branch points between the steps catalyzed by PAL and CHS such that some products are synthesized without any requirement for CHS activity. Thus, differential expression of the two gene families may be attributed to the different needs of the plant tissue for certain compounds at any particular instance.

Our data are in agreement with the model proposed by Van Brussel et al. (1990) for the *V. sativa-R. leguminosarum* symbiosis, in which, after induction of the *nod* genes, the bacteria produce factors that signal the plant to make more flavonoids. At this stage we postulate that the additional flavonoids are involved in maintaining *nod* gene expression while infection progresses. However, flavonoids perform other functions in the plant. One of the functional roles proposed for flavonoids is to act as natural auxin transport inhibitors (Jacobs and Rubery, 1988). Auxin transport inhibitors have been shown to elicit nodule-like structures on alfalfa roots (Hirsch et al., 1989). Some phenylpropanoid derivatives have been shown to have cytokinin-like activities (Binns et al., 1987), and cytokinins have also been implicated in nodule initiation (Long and Cooper, 1988). Hence, it is possible that these secondary flavonoids produced as a result of infection function by affecting hormonal distribution and are involved in cortical cell proliferation.

The induction of the different sets of the PAL and CHS genes in the postinfection stage and during nitrogen stress in nodules formed by Fix^- strains suggests involvement of different signalling mechanisms. Subsets of both the PAL and CHS gene families appear to be induced specifically by the symbiont but not by stress. These enzymes catalyze the same reaction irrespective of which gene member is expressed in response to a particular stimulus. Thus, induction of specific members must be spatially localized in the infected roots. Furthermore, additional levels of regulation may control the production of either flavonoid *nod* gene inducers, flavonoids for modifying hormonal distribution, or phytoalexins. Our future studies will focus on isolating other gene-specific probes for PAL and CHS, which may be useful for spatially localizing the corresponding gene transcripts.

METHODS

Plant Growth and Inoculation

Seeds of soybean (*Glycine max*) cv Prize (Strayer Seed Farms, Hudson, Iowa), and cv Bragg and the derived Bragg mutant *nts*382 (gifts from Dr. P.M. Gresshoff) were surface-sterilized in

10% bleach for 12 min, followed by five to eight rinses in sterile distilled water. Seeds were germinated in pots of sterile vermiculite for 3 days. The seeds were then either inoculated with late log phase *Bradyrhizobium japonicum* or *Rhizobium meliloti* cultures and watered with sterile distilled water, or transplanted (cv Prize) to modified leonard jars (Appelbaum et al., 1986) and inoculated. Plants were watered with a nitrogen-free nutrient solution and grown in growth chambers at 28°C under a 16 hr light:8 hr dark regime. Isolated nodules, uninoculated roots, or nodulated root sections were harvested in liquid nitrogen and stored at −80°C until use.

The *B. japonicum* bacterial strain USDA 110 was used as the wild-type Fix^+ strain. A deletion mutant of USDA 110 in the *nif*KD genes, BJ702 (a gift from the late Dr. B. Chelm), was used as the Nod^+ Fix^- strain. Bacterial cultures of *B. japonicum* for inoculation were grown in yeast extract-mannitol broth at 28°C until late log phase. *R. meliloti* cultures were grown at 28°C until late log phase in modified yeast extract-mannitol broth.

DNA Manipulations

Standard techniques were used (Maniatis et al., 1982) unless otherwise stated. The cDNA clones for French bean (*Phaseolus vulgaris*) chalcone synthase (pCHS1) (Ryder et al., 1984) and phenylalanine ammonia-lyase (pPAL5) (Edwards et al., 1985) were kind gifts from Dr. C. Lamb. cDNA inserts for hybridizations were isolated from agarose gels by the ground-glass procedure (Vogelstein and Gillespie, 1979). Inserts were used to make probes by the random priming method (Feinberg and Vogelstein, 1983).

A cDNA expression library in λgt11 to poly(A) RNA from nodules 14 DPI of cv Prize (Nirunsuksiri, 1990) was screened with cDNA inserts of pPAL5 and pCHS1 according to Maniatis et al. (1982). The cDNA inserts from the hybridizing phage were subcloned into pSP73 or pUC18. Double-stranded plasmids were sequenced with Sequenase ver 2.0 (United States Biochemical) according to the instructions of the manufacturer. The DNA sequence data was analyzed using Microgenie software (Beckman).

Plant DNA Isolation and Analysis

Total genomic DNA was isolated from cv Prize by a modified hexadecyltrimethyl-ammonium bromide procedure (H.E. Richter and C. Sengupta-Gopalan, unpublished results). Restricted DNA was fractionated on a 1.0% agarose gel, blotted onto nitrocellulose, and probed with α-^{32}P-labeled inserts. Prehybridization for 4 hr and hybridization for 16 hr to 20 hr was carried out in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 5 mM sodium phosphate, pH 7.0, 0.1% SDS, 0.1 mg/mL denatured calf thymus DNA, and 0.04 mg/mL poly(A) at 42°C. The nitrocellulose blots were washed three times with 2 × SSC, 0.1% SDS at 42°C for 20 min, followed by one wash with 0.2 × SSC, 0.1% SDS at 42°C for 20 min.

RNA Isolation and Analysis

Polysomal RNA was isolated from nodules and roots of cv Prize (Sengupta et al., 1981). Total RNA was isolated from roots and inoculated roots of cv Bragg and *nts*382 by a LiCl precipitation method (de Vries et al., 1982). Poly(A) RNA was isolated by subjecting the RNA to poly(U)-Sephadex chromatography (Murray et al., 1981). The text refers to polysomal poly(A) RNA and total poly(A) RNA, which means that the poly(A) RNA was isolated either from polysomal RNA or total RNA, respectively.

Poly(A) RNA was fractionated on a 1% formaldehyde agarose gel, blotted onto nitrocellulose, and probed with α-^{32}P-labeled inserts (Thomas, 1980). Prehybridization, hybridization, and washing conditions were the same as for DNA gel blots described above.

Identical conditions were used for the isolation of total and poly(A) RNA from all samples, and RNA was quantified spectrophotometrically in duplicate to avoid errors due to loading variations. To compare hybridization signals in RNA gel blots, all samples were electrophoresed in duplicate in the same gel and blotted intact to keep conditions constant. Duplicate blots were probed with either the 3′ probes or 5′ probes for both genes.

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