

# Flavan-Containing Cells Delimit *Frankia*-Infected Compartments in *Casuarina glauca* Nodules<sup>1</sup>

Laurent Laplaze, Hassen Gherbi, Thierry Frutz, Katharina Pawlowski, Claudine Franche, Jean-Jacques Macheix, Florence Auguy, Didier Bogusz, and Emile Duhoux\*

Physiologie Cellulaire et Moléculaire des Arbres, GeneTrop Institut de Recherche pour le Développement, 911 Avenue Agropolis, 34032 Montpellier cedex 1, France (L.L., H.G., T.F., C.F., F.A., D.B., E.D.); Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands (K.P.); Biochemie der Pflanze, Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Untere Karspüle 2, 37073 Göttingen, Germany (K.P.); and Laboratoire de Biotechnologie et Physiologie Végétale Appliquée, Université Montpellier 2, Place Eugène Bataillon, 34095 Montpellier cedex 5, France (J.-J.M.)

---

We investigated the involvement of polyphenols in the *Casuarina glauca*-*Frankia* symbiosis. Histological analysis revealed a cell-specific accumulation of phenolics in *C. glauca* nodule lobes, creating a compartmentation in the cortex. Histochemical and biochemical analyses indicated that these phenolic compounds belong to the flavan class of flavonoids. We show that the same compounds were synthesized in nodules and uninfected roots. However, the amount of each flavan was dramatically increased in nodules compared with uninfected roots. The use of *in situ* hybridization established that chalcone synthase transcripts accumulate in flavan-containing cells at the apex of the nodule lobe. Our findings are discussed in view of the possible role of flavans in plant-microbe interactions.

---

Flavonoids are secondary metabolites derived from the phenylpropanoid pathway. They are involved in various biological processes, including flower pigmentation (anthocyanins) and protection against UV irradiation (Shirley, 1996). Flavonoids play key roles at different levels of plant-microbe interactions (Dixon and Paiva, 1995; Shirley, 1996). In legumes the accumulation of flavonoid compounds, identified as phytoalexins, occurs in response to pathogen attack. These compounds have been shown to prevent the spread of the pathogen (Dixon and Paiva, 1995). Particular flavonoids have also been implicated in the establishment of pathogenic and symbiotic plant-microbe interactions, in particular, in the *Rhizobium*-legume symbiosis (Peters and Verma, 1990). Specific flavonoids released from the roots of legumes interact with the NodD protein of *Rhizobium* to activate transcription of other *nod* genes responsible for the synthesis of Nod factors (Dénarié et al., 1996; Long, 1996). Aside from their role as signal molecules in root exudates, flavonoids might also be involved in the morphogenesis of legume nodules. Since specific flavonoids can inhibit polar auxin transport (Jacobs and Rubery, 1988), they were proposed to change the phytohormone balance during nodule

initiation (Hirsch et al., 1989; Yang et al., 1992; Mathesius et al., 1998). Moreover, Charrier et al. (1998) suggested a role of flavonoids in modulating nutrient exchanges and enzymatic activities in legume nodules. Furthermore, the accumulation of flavonoids in some ineffective symbioses have been related to a host defense response elicited by rhizobia, which could be part of the regulation of nodulation (Grosskopf et al., 1993).

The flavonoid biosynthetic pathway has been extensively studied in legumes (see e.g. Paiva et al., 1991; Maxwell et al., 1993; McKahn and Hirsch, 1994; Charrier et al., 1995; Djordjevic et al., 1997). Chalcone synthase (CHS), which catalyzes the first step of flavonoid biosynthesis, is a key enzyme in this pathway. It condenses three molecules of malonyl CoA with 4-coumaroyl CoA to produce chalcones (Martin, 1993). CHS genes have been cloned from many plants and are often encoded by small gene families (Martin, 1993). Several authors have reported that CHS is a key regulatory enzyme of flavonoid biosynthesis (Hahlbrock and Scheel, 1989) and that the study of CHS gene expression is a good molecular marker of flavonoid production. CHS gene expression is subject to complex developmental and environmental regulation (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). Its expression is induced by nitrogen deprivation in alfalfa (Coronado et al., 1995) and upon inoculation by rhizobia in soybean and vetch (Estabrook and Sengupta-Gopalan, 1991; Recourt et al., 1992). In pea the highest level of *chs* expression was found in uninfected apical parts of the nodule, suggesting a developmental function of flavonoids (Yang et al., 1992).

Whereas rhizobia interact with legumes, *Frankia* strains establish root nodule symbioses with eight different angiosperm families collectively called actinorhizal plants (Benson and Silvester, 1993). The mode of infection depends on the host plant. Two modes of infection of actinorhizal plants have been described: intercellular and intracellular (Berry and Sunell, 1990; Franche et al., 1998). Intracellular infection (e.g. of *Casuarina glauca*) starts with root hair curling induced by an unknown *Frankia* signal. Bacteria penetrate a curled root hair and grow intracellularly. Root hair infection induces limited cell divisions in the cortex, giving rise to the so-called prenodule. *Frankia* infects some

---

<sup>1</sup> This research was supported by the Institut de Recherche pour le Développement.

\* Corresponding author; e-mail duhoux@mpl.ird.fr; fax 33-4-67-63-82-65.

of the prenodule cells. Concomitantly with prenodule formation, cell divisions occur in the pericycle opposite to a protoxylem pole, leading to the formation of a nodule primordium. Its cells become infected by bacterial hyphae coming from the prenodule. The involvement of flavonoids in actinorhizal symbioses is poorly understood. Because of the similarities of the infection process between some actinorhizal plants and legumes, flavonoids were proposed to act as plant signals activating the production of a *Frankia* root hair-deforming factor (Prin and Rougier, 1987; Van Ghelue et al., 1997).

As part of our investigation into plant responses to *Frankia* infection, we characterized the major polyphenol compounds and monitored *chs* gene expression in *C. glauca* nodules. We report a cell-specific accumulation of phenolic compounds in cell layers delimiting *Frankia*-infected areas of the nodule cortex. Histochemical and biochemical analyses indicated that compounds found in these cell strands belong to the flavan group of flavonoids. We also report that high levels of CHS transcripts were found in the uninfected tannin-containing cells at the apex of the nodule lobes. Finally, we discuss the possible functions of flavans in the *C. glauca*-*Frankia* interaction.

## MATERIALS AND METHODS

### Plant Material

*Casuarina glauca* plants were grown in a greenhouse and inoculated with *Frankia* as previously described (Gherbi et al., 1997). Mature nodules (three to six lobes) from 5- to 6-month-old plants were harvested 3 to 4 weeks after inoculation and used for the experiments.

### Histochemistry

Plant material was fixed and embedded in paraffin as described previously (Gherbi et al., 1997). Sections (7  $\mu$ m) were cut with a microtome (Jun GRM 2055, Leica Microsystems, Wetzlar, Germany). After staining, sections were mounted in the staining reagent or in glycerine plus water (15%, v/v) and examined with a light microscope (model DMR13, Leica). Two filter sets were used: a UV filter set with a 340- to 380-nm excitation and a 425-nm barrier filter, and a blue filter set with a 450- to 490-nm excitation filter and a 515-nm barrier filter. For the structural study, sections were stained with 0.025% (w/v) toluidine blue.

Flavonoid compounds were detected with Neu's reagent (Neu, 1956). Sections were immersed in 1% (w/v) 2-aminoethyl-diphenyl borinate (Fluka, Milwaukee, WI) in absolute methanol for 2 to 5 min, mounted in glycerine water, and observed with epifluorescence. The results were confirmed with Wilson's reagent (Hariri et al., 1991).

A reagent of vanillin-HCl (Sarkar and Howarth, 1976) was employed for analysis of catechins and condensed tannins. Sections were immersed in 10% (w/v) vanillin in 1 volume of absolute ethanol mixed with 1 volume of concentrated HCl, mounted in this reagent, and observed with a light microscope. Results were confirmed using 4-dimethyl-aminocinnamaldehyde staining (Feucht and

Treutter, 1990) on handmade thick sections. Hydrolyzable tannins were detected according to the method of Schneider (1977).

### Extraction and Analysis of Soluble Phenolics

Plant material was ground in cold ethanol:water (4:1) containing 2% (w/v)  $K_2SO_3$ . The extract was agitated for 15 min at 4°C and vacuum filtered. This step was repeated three times. After ethanol evaporation, the aqueous phase was extracted four times with EtOAc containing 20% (w/v)  $(NH_4)_2SO_4$  and 2% (w/v) metaphosphoric acid. The organic phase was then reduced to dryness in vacuo and the residue was dissolved in MeOH. Root and nodule extractions were repeated two times using different tissue samples.

Methanolic extracts were analyzed by HPLC. The liquid chromatograph (model 600E, Waters, Milford, MA) was equipped with a 5-mm  $C_{18}$  column (250  $\times$  5 mm; Spherisorb, Machery and Nagel, Düren, Germany). The following linear gradient elution system was applied at a flow rate of 1 mL  $min^{-1}$ : within 13 min from 7% to 15% solvent B (acetonitrile) in solvent A (water), within 17 min at 15% B, within 5 min from 15% to 19% B, within 15 min at 19% B, within 5 min from 19% to 27% B, within 5 min at 27% B, within 5 min at 80% B, within 5 min from 80% to 100% B. Injections of 5  $\mu$ L were carried out using an automatic sampler (model U6K, Waters). Detection of compounds was done photometrically by a photodiode array detector (model 990, Waters). (+)-Catechin and (-)-epicatechin were identified and quantified by chromatographic and spectrophotometric comparison with standards from Extrasynthese (Genay, France) and Sigma (St. Louis), respectively. Other flavans were expressed as (+)-catechin equivalents from internal standardization.

### Isolation of a *C. glauca* CHS cDNA Clone

A *lgt10* cDNA library was prepared from *C. glauca* root nodule RNA as described elsewhere (Gherbi et al., 1997). The library was screened by differential hybridization of randomly picked clones with biotin-labeled root or nodule cDNA (Gherbi et al., 1997). The insert of *cgCHS1* was subcloned into pGEM-T vector (Promega, Madison, WI). Both strands of the insert were sequenced using an automatic sequencing system (model 373A, Applied Biosystems, Foster City, CA). Sequence data were analyzed by the BLAST program (Altschul et al., 1990).

### Southern-Blot Analysis

Genomic DNA of *C. glauca* was isolated using a plant mini kit (Dneasy, Qiagen, Courtaboeuf, France) according to the manufacturer. For Southern-blot analysis, 10  $\mu$ g of DNA was cut by *Bam*HI and *Hind*III, separated on an 0.8% (w/v) agarose gel, and blotted as described previously (Gherbi et al., 1997). Prehybridization and hybridization were performed at 65°C using 6 $\times$  SSC, 5 $\times$  Denhardt's solution, 1% (w/v) SDS, and 0.1 mg/mL of salmon-sperm DNA. The insert of *cgCHS1* was labeled with [ $^{32}$ P]dCTP

using an oligolabeling kit (Pharmacia, Piscataway, NJ). After hybridization the filters were washed at 65°C successively using 2× SSC, 0.1% SDS (twice for 20 min), 1× SSC, 0.1% SDS (twice for 20 min), and 0.5× SSC, 0.1% SDS (twice for 20 min).

### Isolation of Total RNA and Reverse Transcription (RT) PCR Analysis

Total RNA was isolated from nodules, uninfected roots, and stems/leaves using the procedure of Bugos et al. (1995). Total RNA (2.5 µg) was reverse transcribed using an mRNA capture kit (Boehringer Mannheim, Basel) to avoid DNA contamination in a total volume of 50 µL as described by the manufacturer. The reaction mixture was incubated at 42°C for 1 h and then for 5 min at 94°C. The PCR reactions were performed with 5 µL of the cDNA solution in 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 0.2 mM each dNTPs, 0.2 mM of each primer, and 1 unit of *Taq* polymerase (Promega) in a total volume of 50 µL. A 745-bp fragment was amplified using two primers with sequence similarity to the *cgCHS1* coding region (forward: 5'-CTGTCTCGACCAAAGCAC-3', reverse: 5'-TGAGATGAGCCCAGGAAC-3') within 35 cycles (94°C, 1 min; 55°C, 1 min; and 72°C, 1 min). As an internal control, PCR was performed simultaneously using ubiquitin primers (Horvath et al., 1993). PCR products (5 µL) were separated by electrophoresis on a 1.6% (w/v) agarose gel, followed by blotting onto a positive membrane (Appligene Oncor, Heidelberg). The *cgCHS1* or ubiquitin products were detected by hybridization with <sup>32</sup>P-labeled inserts of the corresponding cDNA clones. Control reactions in which RNA was treated as above but without reverse transcriptase gave no signal after hybridization. RT-PCR experiments were repeated using at least two independent RNA preparations.

### In Situ Localization of *cgCHS1* mRNA

In situ hybridization was performed as previously described (Gherbi et al., 1997). For generation of an RNA probe, *cgCHS1* was amplified with two primers homologous to the coding region (5'-TAGTGGTGTGGACATGCC-3' and 5'-TGAGATGAGCCCAGGAAC-3'). The 435-bp product was cloned in pGEM-T (Promega). Two clones were selected, resulting in plasmids pGEM/CHS26 (antisense) and pGEM/CHS2 (sense). For antisense and sense RNA production, pGEM/CHS26 and pGEM/CHS2 were cut with *Pst*I and in vitro transcribed by T7 polymerase. Radioactive labeling was performed as previously described (Gherbi et al., 1997).

## RESULTS

### Histological Study of *C. glauca* Nodule Compartmentalization

Actinorhizal nodules consist of multiple lobes, each representing a modified lateral root without a root cap, with a superficial periderm and infected cells in the expanded

cortex. Longitudinal sections of mature *C. glauca* nodules stained with toluidine blue showed the presence of large amounts of phenolic compounds (green color in Fig. 1, A–C). These compounds were abundant in endodermal cells and in a few layers of cortical cells below the periderm. In the cortex, cells containing phenolics formed continuous files from the apex to the base of the nodule lobe (Fig. 1, A–C). These uninfected cells containing phenolics bound layers of infected and uninfected cells (Fig. 1, A and B). In each layer, *Frankia* grew acropetally and never crossed files of phenolic-filled cells (Fig. 1, A and B) except during early layer infection (Fig. 1C). In this last case, hyphae went through a phenolic-free cell at the base of the file. Transverse sections of *C. glauca* nodule lobes show that cortical cells accumulating phenolics are organized in concentric layers (data not shown).

An endophyte-free nodule root is formed at the apex of a nodule lobe. This root has specific features (i.e. agravitropism, aerenchyma), and is thought to facilitate oxygen access to the nodule lobe. Figure 1A shows the presence of phenolics in cells located at the boundary between the nodule lobe apex and the nodule root.

Taken together, these results show accumulation of phenolic compounds in the endodermis, below the periderm, and in cortical cells compartmentalizing the infected cortical tissue. In the cortical tissue, continuous layers of phenolic-containing cells separate layers containing both infected and uninfected cells. The boundary between the apex of the infected nodule lobe and the uninfected nodule root is also marked by a few layers of phenolic-containing cells. Thus, accumulation of phenolics creates a compartmentation of *C. glauca* root nodules. Nearly cylindrical layers of cells accumulating phenolics limit different cortical areas where *Frankia* infection takes place.

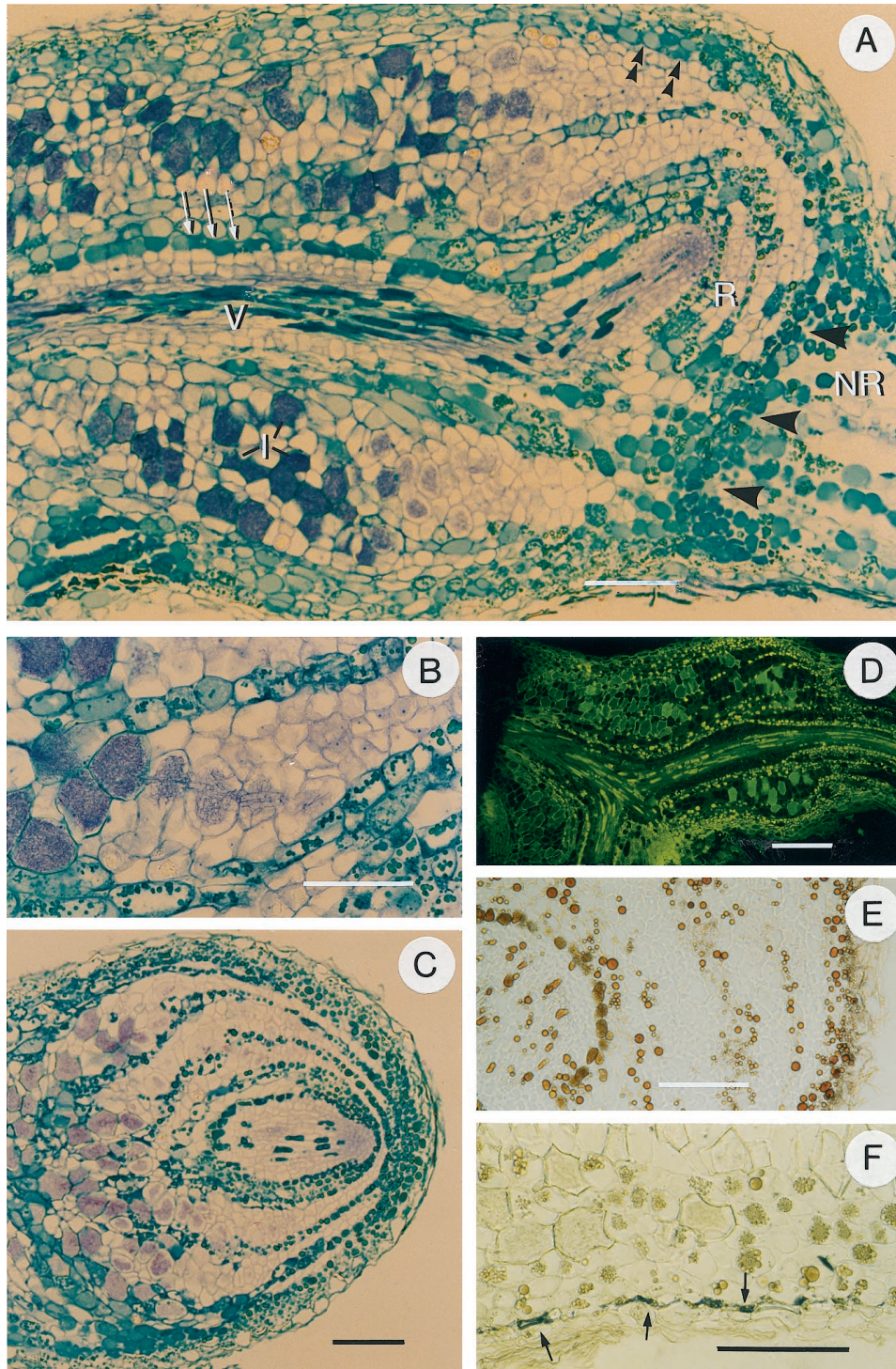
### Histochemistry of *C. glauca* Nodule Phenolics

To characterize phenolic compounds involved in this compartmentation process, histochemical analyses were conducted on sections of nodule lobes. Autofluorescence and histochemical data are listed in Table I.

White-blue autofluorescence was seen in the cell walls of periderm and endodermal cells, while a white-yellow fluorescence was detected in infected cell walls after excitation at 365 nm, indicating the presence of lignin and/or suberin. The color of the infected cell walls suggested that other phenolics might also be present. These findings are in agreement with data published by Berg and McDowell (1987) on the cell wall cytochemistry of *Frankia*-infected cells in *C. glauca* nodules.

Weak orange autofluorescence after excitation at 365 nm and bright yellow fluorescence after excitation at 420 nm were observed in phenolic-containing cells. Treatment with either Neu's or Wilson's reagent (Fig. 1D) gave the same results. These tests suggested the presence of flavonoids, presumably flavan derivatives that are known to be poorly fluorescent under UV light. This hypothesis was confirmed by using vanillin-HCl and 4-dimethyl-aminocinnamaldehyde reagents. Positive reactions were found for both reagents in all cells containing flavonoids (Fig. 1E), confirming that





**Figure 1.** Localization and histochemical characterization of *C. glauca* nodule phenolics. A, Longitudinal section of a nodule lobe stained with toluidine blue showing a central vascular bundle (V), a ramification of the nodule lobe (R), and the basis of the nodular root (NR). Purple cells are *Frankia*-infected cells (I). Phenolics (green) accumulate in the endoderm (white arrows), below the periderm (double black arrowheads), and between the nodule lobe and the nodular root (black arrowheads). Bar = 100  $\mu\text{m}$ . B, Detail of a longitudinal section of a nodule lobe stained with toluidine blue showing *Frankia* infecting acropetally a cortical compartment surrounded by phenolics containing cells arranged in files. Bar = 100  $\mu\text{m}$ . C, Longitudinal section of a young nodule lobe stained with toluidine blue showing cortical files of cells accumulating phenolic compounds. *Frankia* infection progresses acropetally in each layer. Bar = 100  $\mu\text{m}$ . D, Longitudinal section of a nodule lobe stained with Wilson's reagent showing that flavonoids accumulate in the endoderm, below the periderm, and in the cortical cell files. Bar = 200  $\mu\text{m}$ . E, Transversal section of a nodule lobe stained with vanillin-HCl reagent. Positive reaction (red color) is found within phenolic globules. Bar = 100  $\mu\text{m}$ . F, Detail of a longitudinal section of a nodule lobe stained with Schneider's reagent. A positive reaction (black deposit) is found just below the periderm (arrows). Bar = 100  $\mu\text{m}$ .

**Table 1.** Histochemistry of phenolic compounds in *C. glauca* nodules

Reagents	Phenols	Staining <sup>a</sup>	Sites of Reaction <sup>b</sup>
Autofluorescence	Flavonoids	**Yellow (blue light) *Orange (UV light) **Blue-white (blue light) **White-yellow (blue light)	E, C, S E, C, S E, P I
Neu	Flavonoids	**Yellow (blue light) *Orange (UV light) **Blue-white (blue light) **White-yellow (blue light)	E, C, S E, C, S E, P I
Wilson	Flavonoids	**Yellow (blue light) *Orange (UV light) **Blue-white (blue light) **White-yellow (blue light)	E, C, S E, C, S E, P I
Vanillin-HCl	Condensed tannins	**Red-brown	E, C, S
4-Dimethyl-amino-cinnamaldehyde	Condensed tannins	**Blue	E, C, S
Schneider	Hydrolyzable tannins	**Dark deposit	E, S

<sup>a</sup> \*\*, Strong reaction; \*, moderate reaction.  
<sup>b</sup> E, Endoderm; C, cortical cell strands; S, subperidermal cells; P, periderm; I, infected cells.

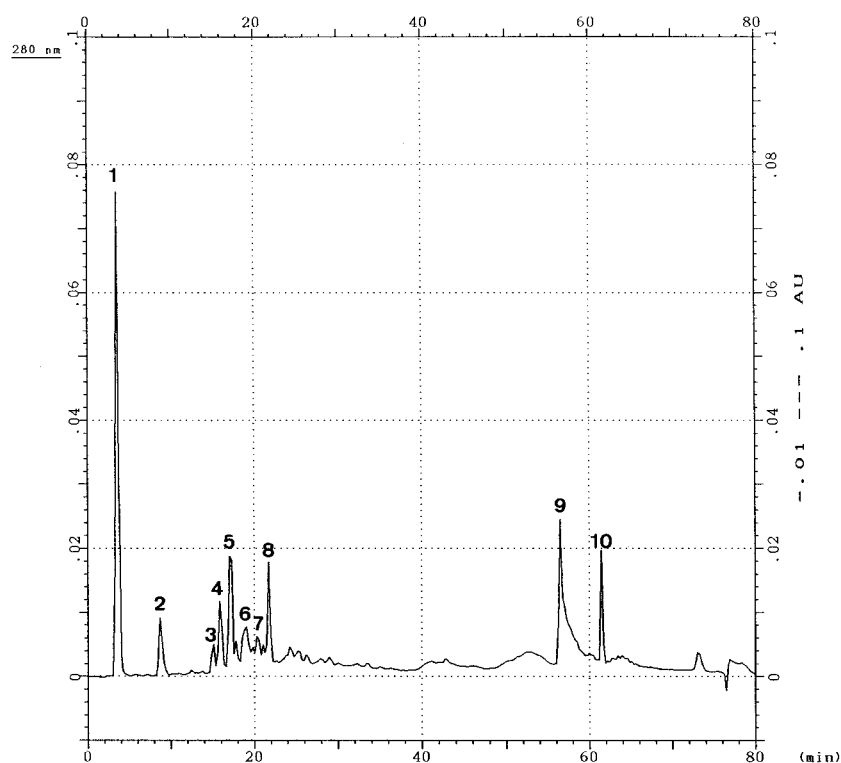
flavans are present in these cells. These flavans may be both monomeric flavan-3-ols (catechins) and oligomeric and polymeric forms (condensed tannins). Dark staining with Schneider's reagent (Fig. 1F) was detected in cell layers just below the periderm and endodermis, suggesting that these cells also contain hydrolyzable tannins.

Histochemical analyses suggest that condensed tannins (flavans) are the major components of the deposits of phenolic compounds found in uninfected nodule cells. Gallic tannins accumulated specifically at the boundaries of

the infected cortex, i.e. in the endodermis and below the periderm.

#### HPLC Analysis of Root and Nodule Phenolic Compounds

We further characterized soluble phenolics from *C. glauca* nodules using HPLC. Alcoholic extracts of nodules were analyzed on a reverse-phase C<sub>18</sub> HPLC column. As shown in Figure 2, HPLC analysis of nodule extracts showed 10 major peaks. Spectral data indicated that the



**Figure 2.** HPLC analysis of *C. glauca* nodule phenolics analyzed at 280 nm. Products 5 and 6 were identified as (+)-catechin and (-)-epicatechin, respectively. The amounts of flavonoids were calculated from the area of the peaks detected by analytical HPLC.



**Table II.** Flavonoid content of roots and nodules

Peaks 5 and 8 were identified as soluble (+)-catechin and (–)-epicatechin, respectively. All other peaks are catechins belonging to the flavan class of flavonoids.

Plant Material	Compound							
	3	4	5	6	7	8	9	10
	<i>μmol catechin equivalents g<sup>-1</sup> fresh wt</i>							
Root	0.027	0.077	0.129	0.055	0.027	0.134	0.331	0.647
Nodule	0.274	0.807	1.712	0.617	0.214	0.963	2.905	0.954

two first peaks were not phenolic compounds (data not shown). Based on retention time, co-injection experiments, and absorption spectra, peaks 5 and 8 were identified as soluble (+)-catechin and (–)-epicatechin, respectively. All other compounds presented the same UV spectrum as catechins, with a maximum at 280 nm, suggesting that they belong to the flavan class of flavonoids. Because of their long retention time, peaks 9 and 10 might be polymerized forms of flavan (i.e. condensed tannins).

To compare flavonoid metabolism in nodules and uninfected roots, the same biochemical studies were conducted with root material. The same profile was observed in roots as in nodules (data not shown). Products 3 to 10 were quantified by integration and are expressed as catechin equivalents. Table II shows that there was a strong increase in flavan content on nodules compared with uninfected roots. These results indicate that nodules accumulated the same compounds as roots but in a greater amount. Moreover, there were changes in the relative proportion of

products: while in uninfected roots compound 10 was the most abundant, compound 9 was the major component in nodules. Also, (+)-catechin and (–)-epicatechin amounts were similar in roots, whereas (+)-catechin was about two times more abundant in nodules. This indicated some subtle reorientations of metabolic fluxes in the different pathways.

**cgCHS1: Isolation and Analysis of Expression in *C. glauca* Nodules**

Since the synthesis and accumulation of secondary metabolites can take place at different sites (Reinold and Hahlbrock, 1997), and since flavans are derived from the normal flavonoid pathway, we chose a CHS cDNA clone as a marker of flavonoid production to determine in which cell type flavan synthesis occurs.

The cDNA clone corresponding to a CHS gene was isolated from a *C. glauca* nodule cDNA library as described

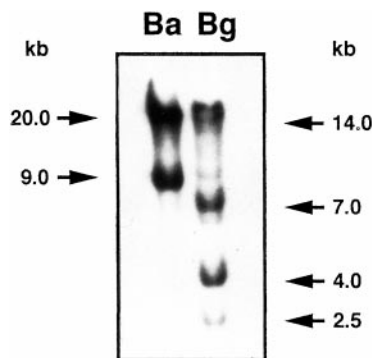


**Figure 3.** Sequence alignment of the chalcone synthase cDNAs from *C. glauca* (*cgCHS1*), *Juglans* sp. (*J. nigra* × *J. regia*) (*JSPCHS1*; accession no. X94995), and *V. unguiculata* (*VUCHSCH*; accession no. X74821). Shaded boxes indicate conserved nucleotides. Putative initiation and termination codons are marked by boxes.

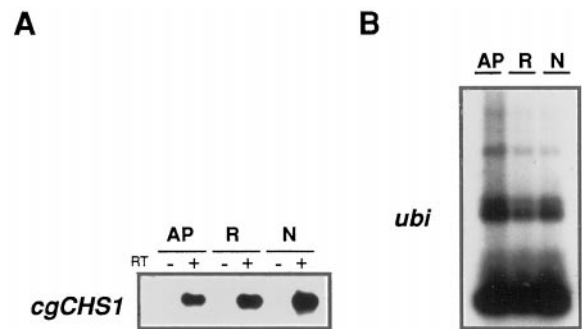
previously (Gherbi et al., 1997). This cDNA, designated *cgCHS1*, was 1,407 bp long with an ORF of 1,170 bp corresponding to 389 amino acids (accession no. AJ132323). A search of the nucleic acid and protein databases showed high sequence similarity to *CHS* genes of other plants. For example, the ORFs in *Juglans* sp. (*J. nigra* × *J. regia*) JSPCHS1 and *Vigna unguiculata* VUCHSCH showed a high degree of similarity to the sequence of *cgCHS1*: 84% and 72% at the nucleotide level and 91% and 83% identity at the amino acid level (Fig. 3), respectively.

To determine the number of *CHS* genes in the *C. glauca* genome, DNA was digested with *Bam*HI and *Hind*III, and a Southern transfer experiment was performed with the entire *cgCHS1* cDNA clone as a probe. Several bands were detected with both enzymes (Fig. 4) suggesting that there are several *CHS* genes in *C. glauca*, as in other plants (Martin, 1993).

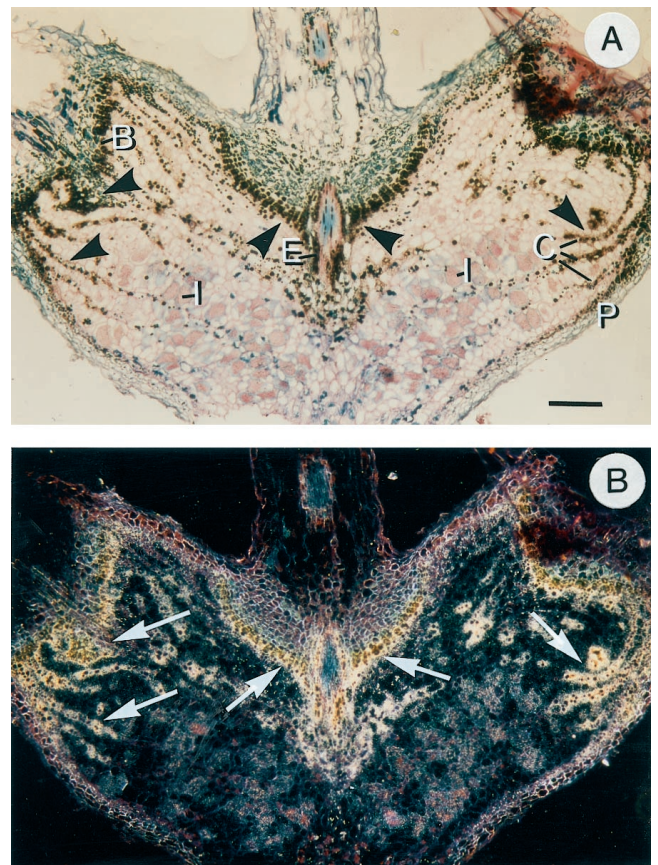
We were unable to detect *cgCHS1* transcripts using the northern blot technique. Therefore, RT-PCR, a more sensitive technique, was used to determine the expression of *cgCHS1*. As shown in Figure 5A, a 745-bp fragment was amplified from aerial parts, uninfected roots, and nodule RNA. Thus, *cgCHS1* is expressed in all tested organs. No PCR product was observed when the reverse transcription step was omitted (Fig. 5A), indicating the absence of any DNA contamination. PCR on genomic DNA using the same primers amplified a product of about 1,100 bp, suggesting that at least one intron was present in the amplified fragment (data not shown). *cgCHS1* transcripts were localized in *C. glauca* nodules by in situ hybridization with sense and antisense RNA probes. As shown in Figure 6, *cgCHS1* mRNA is present in phenolic-containing cell layers between the nodule lobe and the nodule root, below the periderm, in the endoderm, and in the apical part of the cortical cell files. *cgCHS1* mRNA was also detected at the apex of young nodule lobes before nodule root formation (data not shown). Thus, in situ hybridization studies showed that the expression of *cgCHS1* occurred in cells where flavans were detected. However, *cgCHS1* expression was restricted to the apical part of the cortical flavonoid-containing cell layers, suggesting that in the cortex, flavonoid biosynthesis is restricted to these cells. Low levels of *CHS* transcripts were also present in infected cells.



**Figure 4.** Southern-blot hybridization of genomic DNA isolated from *C. glauca*. Ten micrograms of DNA was digested with *Bam*HI (Ba) and *Bgl*II (Bg) and probed with  $^{32}$ P-labeled *cgCHS1* cDNA.



**Figure 5.** RT-PCR analysis of *cgCHS1* expression in *C. glauca* aerial parts (AP), uninfected roots (R), and nodules (N). A, *cgCHS1* expression was found in all tissue tested. No amplification was found when the reverse transcription step was omitted. B, Amplification of ubiquitin RNA used as an internal control.



**Figure 6.** Localization of *CgCHS1* transcripts in *C. glauca* nodules. Shown are bright- (A) and dark-field (B) micrographs of 7- $\mu$ m sections hybridized with  $^{35}$ S-labeled antisense transcripts. Arrowheads on the bright-field micrograph indicate the locations of tannin accumulation. White grains on the dark-field micrograph (highlighted by white arrows) indicate the locations of hybridizing  $^{35}$ S-labeled transcripts. E, Endodermis; P, periderm; C, cortical cell strands; B, boundary between the nodule lobe and the nodule root; I, infected cells. Bar = 100  $\mu$ m.



No signal was found in hybridizations with sense CHS RNA (data not shown).

## DISCUSSION

We have shown that in *C. glauca* nodules, *Frankia*-infected cells occur in layers surrounded by tannin-containing cell layers located below the periderm, in the endodermis, and in the cortex. This characteristic distribution of phenolics was also observed in the early steps of nodule development, i.e. in the pre-nodule and in the nodule primordia (data not shown). Interestingly, GUS reporter gene expression driven by the CaMV 35S promoter in transgenic *C. glauca* nodules within the nodule cortex was limited to tannin-containing cells, confirming the specific behavior of these cells (C. Franche, unpublished data). Thus, in addition to infected and uninfected cells, cells containing phenolics represent a third specialized cell type in the cortex of *C. glauca* nodules.

No such organized tannin accumulation is found in *C. glauca* pseudonodules induced by auxin transport inhibitors (E. Duhoux, unpublished data). Regular layers of phenolic-containing cells in the nodule cortex are also found in other actinorhizal nodules, e.g. those of *Alnus glutinosa*. There, the regular pattern of layers of cells containing phenolics is absent in ineffective nodules induced by *Frankia* strains not capable of symbiotic nitrogen fixation (Guan et al., 1996). Therefore, the existence of these cell layers seems to be the result of signal exchange with the endophyte. However, this compartmentation is initiated prior to infection of the nodule lobe and therefore is not solely a response to *Frankia*.

A similar accumulation of phenolics has previously been described for the early steps of the intracellular infection process in *Casuarina cunninghamiana* (Torrey, 1976), *Comptonia peregrina* (Callahan and Torrey, 1977), and *Alnus glutinosa* (Angulo Carmona, 1974). Furthermore, accumulation of phenolics was also observed during intercellular infection of *Eleagnus angustifolia* (Miller and Baker, 1985) and *Parasponia rigida* (Lancelle and Torrey, 1984), the only non-legume nodulated by *Rhizobium*, suggesting that this phenomenon is a common feature in non-legume root nodules.

In some legume systems, it has been shown that proanthocyanidins (condensed tannins) are present in the root cortex (Stafford, 1997). In *Medicago* spp., flavonoids are detected in the cortex and in the nodule meristem (Charrier et al., 1998). Furthermore, in *Acacia mangium*, a legume tree, polyphenols accumulate at the site of root hair infection, and the developing nodule primordium is surrounded by cortical cells containing large amounts of polyphenols (Y. Prin and E. Duhoux, unpublished data). Therefore, the accumulation of phenolics is not restricted to the root nodules of non-legumes, but seems to play a larger role in woody than in herbaceous plants.

HPLC elution profiles of root and nodule extracts gave the same 10 components. Histochemical and biochemical analyses revealed that all soluble phenolic compounds in nodules belong to the flavan class of flavonoids. Our data indicate that (+)-catechin and (-)-epicatechin are the ma-

ior monomeric flavans, and that polymerized flavans (i.e. condensed tannins) are also present (Fig. 2, peaks 9 and 10). Although in the alcoholic extract, flavans were the only soluble flavonoids detected, cell wall-bound flavonoid compounds might also be located within the cell walls of *Frankia*-infected cells, as suggested by autofluorescence and histochemical data. The observed increase in flavan content in nodules and the different proportions of the different compounds in root and nodule suggest that *Frankia* infection stimulates the expression of genes encoding flavan-biosynthetic enzymes. However, recent results from Guo et al. (1998) showed that in the soybean cell-suspension-culture system, posttranscriptional mechanisms are responsible for isoflavonoid phytoalexin accumulation. Identification of the flavan monomers and oligomers found in nodule extracts and the isolation of *C. glauca* genes involved in their biosynthesis will help us to gain an understanding of the regulatory mechanisms responsible for the symbiotic induction of flavan production.

High amounts of phenolics in actinorhizal nodules might restrict the endophyte to certain regions in the cortex, preventing its spread in the vascular tissue, periderm, and meristem. Moreover, it has been shown that phenolics can influence *Frankia* growth in vitro (Perradin et al., 1982). Therefore, it will be interesting to determine whether flavans isolated from *C. glauca* nodules can influence the growth of *C. glauca*-infective *Frankia* strains. Weiss et al. (1997) reported an accumulation of phenylpropanoids in larch mycorrhizae. They observed a significant accumulation of (+)-catechin and (-)-epicatechin in the inner cortex, endodermis, and root apex that remained free of fungi. Since (+)-catechin and (-)-epicatechin have been shown to inhibit fungal growth, they suggested that this accumulation might control fungal invasion. In the legume *Lotus corniculatus*, flavans accumulate in cells arranged in files near the root vascular bundle (Stafford, 1997). Altogether, these data suggest that flavan synthesis is a constitutive plant defense response in roots of *C. glauca*, and that *Frankia* infection increases this reaction. This increase might be mediated by a symbiosis-specific regulation of genes of the flavan biosynthetic pathway. Alternatively, these polyphenol layers might contribute to the protection against secondary infection and/or limit oxygen penetration in the nodule.

Several authors have suggested that flavonoid and isoflavonoid biosynthesis in response to biotic or abiotic stresses is regulated via CHS expression (Martin, 1993). The localization of CHS mRNA by in situ hybridization indicated that *cgCHS1* is expressed in the flavan-containing cells of the apex of the nodule lobe. This expression pattern was correlated with the detection of flavans in the same cell type. This observation implies that flavonoid synthesis depends on the developmental stage of the cortical cells. The inability to detect *cgCHS1* transcripts on northern blot might be due to the fact that *cgCHS1* is expressed transiently in a few specialized cells of the apex. Interestingly, in parsley, several enzymes of the flavonoid pathway appeared much more stable than their corresponding mRNAs (Reinold and Hahlbrock, 1997).



It is therefore possible that in *C. glauca* nodules *chs* expression appears transiently at the apex and is still active in cell derivatives within the nodule lobe to produce the tannin-containing cell layers. This hypothesis can be tested using antibodies directed against CgCHS1. A low level of *cgCHS1* expression was also found in the infected cortical cells. Similarly, flavonoid biosynthetic mRNAs were detected in cells of *Medicago truncatula* colonized by the mycorrhizal fungus *Glomus versiforme* (Harrison and Dixon, 1994). In this case, it was clearly demonstrated that expression was not linked to a defense response. It was proposed that flavonoids might influence carbon metabolism in these cells. As suggested by our histochemical data, *chs* expression in infected cells of *C. glauca* nodules might be associated with the presence of flavonoids in their cell walls. These wall-bound flavonoids have been suggested to function in limiting oxygen access to the places of nitrogen fixation (Berg and McDowell, 1987; Silvester et al., 1990).

In conclusion, during *C. glauca* nodule formation, cell-specific flavan biosynthesis and accumulation delimit cortical compartments containing *Frankia*-infected cells and might restrict endophyte invasion. Recent reports on mycorrhiza-induced biosynthesis and tissue-specific accumulation of phenolics in larch (Weiss et al., 1997) suggest that the same type of strategy is used to control ectomycorrhizal interaction. However, it should be pointed out that catechin and epicatechin accumulate mainly in the endodermis of the mycorrhizal roots or in the calyptra surrounding the root tips, and not in the vicinity of the symbiotic cells (Weiss et al., 1997). The fact that high amounts of phenolics are also found in root nodules of *P. rigida* and *A. mangium* and not in nodules of herbaceous legumes suggests that the woody nature of the host plant is an important factor.

Layers of polyphenol-containing cells lead to a compartmentation of the cortex of the nodule lobe that is composed of several infected areas delimited by flavan-containing cell layers. The most recently infected compartment seems to be the innermost one. The meaning of this second compartmentation is not understood, but obviously some signal exchange with the endophyte is needed for its development.

#### ACKNOWLEDGMENTS

We acknowledge Dr. M. Nicole (Institut de Recherche pour le Développement-GeneTrop, Montpellier, France) and Dr. C. Andary (Faculté de Pharmacie, Montpellier, France) for helpful discussions. We thank Magali Derroja for help with biochemical analysis.

Received February 3, 1999; accepted June 7, 1999.

#### LITERATURE CITED

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410
- Angulo Carmona AF (1974) La formation des nodules fixateurs d'azote chez *Alnus glutinosa*. *Acta Bot Neerl* **23**: 257–303
- Benson DR, Silvester WB (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol Rev* **57**: 297–319
- Berg RH, McDowell L (1987) Cytochemistry of the wall of infected cells in *Casuarina* actinorhizae. *Can J Bot* **66**: 2038–2047
- Berry AM, Sunell LA (1990) The infection process and nodule development. In CR Schwintzer, JD Tjepkema, eds, *The Biology of Frankia and Actinorhizal Plants*. Academic Press, San Diego, pp 61–81
- Bugos RC, Chiang VL, Zhang XH, Campbell ER, Podilla GK, Campbell WH (1995) RNA isolation from plant tissues recalcitrant to extraction in guanidine. *Biotechniques* **19**: 734–737
- Callaham D, Torrey JG (1977) Prenodule formation and primary nodule development in roots of *Comptonia* (*Myricaceae*). *Can J Bot* **51**: 2306–2318
- Charrier B, Coronado C, Kondorosi A, Ratet P (1995) Molecular characterization and expression of alfalfa (*Medicago sativa* L.) flavanone-3-hydroxylase and dihydroflavonol-4-reductase encoding genes. *Plant Mol Biol* **29**: 773–786
- Charrier B, Trinh H, Poirier S, Kondorosi A, Ratet P (1998) Flavanone-3-hydroxylase (F3H) expression and flavonoid localization in nodules of three legume plants reveal distinct tissue specificities. *Mol Plant-Microbe Interact* **11**: 924–932
- Coronado C, Zuanazzi J, Sallaud C, Quirion JC, Esnault R, Husson HP, Kondorosi A, Ratet P (1995) Alfalfa root flavonoid production is nitrogen regulated. *Plant Physiol* **108**: 533–542
- Denarié J, Debelle F, Promé J-C (1996) Rhizobium lipochitooligosaccharide nodulation factors: signalling molecules mediating recognition and morphogenesis. *Annu Rev Biochem* **65**: 503–535
- Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**: 1085–1097
- Djordjevic MA, Mathesius U, Arioli T, Weinman JJ, Gärtner E (1997) Chalcone synthase gene expression in transgenic subterranean clover correlate with localised accumulation of flavonoids. *Aust J Plant Physiol* **24**: 119–132
- Estabrook EM, Sengupta-Gopalan C (1991) Differential expression of phenylalanine ammonia-lyase and chalcone synthase during soybean nodule development. *Plant Cell* **3**: 299–308
- Feucht W, Treutter D (1990) Flavan-3-ols in trichomes, pistils and phelloderm of some tree species. *Ann Bot* **65**: 225–230
- Franché C, Laplaze L, Duhoux E, Bogusz D (1998) Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. *Crit Rev Plant Sci* **17**: 1–28
- Gherbi H, Duhoux E, Franché C, Pawlowski K, Nassar A, Berry AM, Bogusz D (1997) Cloning of a full-length symbiotic hemoglobin cDNA and *in situ* localization of the corresponding mRNA in *Casuarina glauca* root nodule. *Physiol Plant* **99**: 608–616
- Grosskopf E, Ha DTC, Wigender R, Röhrig H, Szecsi J, Kondorosi E, Schell J, Kondorosi A (1993) Enhanced levels of chalcone synthase in alfalfa nodules induced by a Fix<sup>-</sup> mutant of *Rhizobium meliloti*. *Mol Plant-Microbe Interact* **6**: 173–181
- Guan C, Wolters DJ, van Dijk C, Akkermans ADL, van Kammen T, Bisseling T, Pawlowski K (1996) Gene expression in ineffective actinorhizal nodules of *Alnus glutinosa*. *Acta Bot Gall* **143**: 613–620
- Guo Z-J, Lamb C, Dixon RA (1998) Potentiation of the oxidative burst and isoflavonoid phytoalexin accumulation by serine protease inhibitors. *Plant Physiol* **118**: 1487–1494
- Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annu Rev Plant Physiol Plant Mol Biol* **40**: 347–369
- Hariri B, Sallé G, Andary C (1991) Involvement of flavonoids in the resistance of two poplar cultivars to mistletoe (*Viscum album* L.). *Protoplasma* **162**: 20–26
- Harrison MJ, Dixon RA (1994) Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interactions between roots of *Medicago truncatula* and the mycorrhizal fungus *Glomus versiforme*. *Plant J* **6**: 9–20
- Hirsch AM, Bhuvaneshwari TV, Torrey JG, Bisseling T (1989) Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. *Proc Natl Acad Sci USA* **86**: 1244–1248
- Horvath B, Heidstra R, Lados M, Moerman M, Spaink HP, Promé J-C, van Kammen A, Bisseling T (1993) Lipo-oligosaccharides

- of *Rhizobium* induce infection-related early nodulin gene expression in pea root hairs. *Plant J* **4**: 727–733
- Jacobs M, Rubery PH** (1988) Naturally occurring auxin transport regulators. *Science* **241**: 346–349
- Lancelle SA, Torrey JG** (1984) Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. II. Nodule morphogenesis and symbiotic development. *Can J Bot* **63**: 25–35
- Long SR** (1996) *Rhizobium* symbiosis: Nod factor in perspective. *Plant Cell* **8**: 1651–1668
- Martin CR** (1993) Structure, function, and regulation of the chalcone synthase. *Int Rev Cytol* **147**: 233–283
- Mathesius U, Schlaman HRM, Spaik HP, Sautter C, Rolfe BG, Djordjevic MA** (1998) Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. *Plant J* **14**: 23–34
- Maxwell CA, Harrison MJ, Dixon RA** (1993) Molecular characterization and expression of alfalfa isochlorogenicin 2'-O-methyltransferase, an enzyme specifically involved in the biosynthesis of an inducer of *Rhizobium meliloti* nodulation genes. *Plant J* **4**: 971–981
- McKahnn H, Hirsch A** (1994) Isolation of chalcone synthase and chalcone isomerase cDNAs from alfalfa (*Medicago sativa* L.): highest transcript levels occur in young roots and root tips. *Plant Mol Biol* **24**: 767–777
- Miller IM, Baker DD** (1985) The initiation, development and structure of root nodules in *Eleagnus angustifolia* L. (*Elaeagnaceae*). *Protoplasma* **128**: 107–119
- Neu R** (1956) A new reagent for differentiating and determining flavones on paper chromatograms. *Naturwissenschaften* **43**: 82
- Paiva N, Edwards R, Sun Y, Hrazdina G, Dixon R** (1991) Stress responses in alfalfa (*Medicago sativa* L.). 11. Molecular cloning and expression of alfalfa isoflavone reductase, a key enzyme of isoflavonoid phytoalexin biosynthesis. *Plant Mol Biol* **17**: 653–667
- Perradin Y, Mottet MJ, Lalonde M** (1982) Influence of phenolics on *in vitro* growth of *Frankia* strains. *Can J Bot* **61**: 2807–2814
- Peters NK, Verma DPS** (1990) Phenolic compounds as regulators of gene expression in plant-microbe interaction. *Mol Plant-Microbe Interact* **3**: 4–8
- Prin Y, Rougier M** (1987) Preinfection events in the establishment of *Alnus-Frankia* symbiosis: study of the root hair deformation step. *Plant Physiol* **6**: 99–106
- Recourt K, van Tunen AJ, Mur LA, van Brussel AAN, Lugtenberg BJJ, Kijne JW** (1992) Activation of flavonoid biosynthesis in roots of *Vicia sativa* subsp. *nigra* plants by inoculation with *Rhizobium leguminosarum* biovar *viciae*. *Plant Mol Biol* **19**: 411–420
- Reinold S, Hahlbrock K** (1997) *In situ* localization of phenylpropanoid biosynthetic mRNAs and proteins in parsley (*Petroselinum crispum*). *Bot Acta* **110**: 431–443
- Sarkar SK, Howarth RE** (1976) Specificity of the vanillin test for flavanols. *J Agric Food Chem* **24**: 317–320
- Schneider H** (1977) Indicator hosts for pear decline: symptomatology, histopathology, and distribution of mycoplasma-like organisms in leaf veins. *Phytopathology* **67**: 592–601
- Shirley BW** (1996) Flavonoid biosynthesis: "new" functions for an "old" pathway. *Trends Plant Sci* **1**: 377–381
- Silvester WB, Harris SL, Tjepkema JD** (1990) Oxygen regulation and hemoglobin. In CR Schwintzer, JD Tjepkema, eds, *The Biology of Frankia and Actinorhizal Plants*. Academic Press, San Diego, pp 157–174
- Stafford HA** (1997) Roles of flavonoids in symbiotic and defense functions in legume roots. *Bot Rev* **63**: 27–39
- Torrey JG** (1976) Initiation and development of root nodules of *Casuarina* (*Casuarinaceae*). *Am J Bot* **63**: 335–345
- Van Ghelue M, Lovaas E, Ringo E, Solheim B** (1997) Early interactions between *Alnus glutinosa* and *Frankia* strain ArI3: production and specificity of root hair deformation factor(s). *Physiol Plant* **99**: 579–587
- Weiss M, Mikolajewski S, Peipp H, Schmitt U, Schmidt J, Wray V, Strack D** (1997) Tissue-specific and development-dependent accumulation of phenylpropanoids in larch mycorrhizas. *Plant Physiol* **114**: 15–27
- Yang WC, Canter Cremers HCJ, Hogendijk P, Katinatis P, Wijffelman CA, Franssen H, van Kammen A, Bisseling T** (1992) *In situ* localization of chalcone synthase mRNA in pea root nodule development. *Plant J* **2**: 143–152