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# A Type-I Chalcone Isomerase mRNA Is Highly Expressed in the Root Nodules of *Elaeagnus umbellata*

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**We have used the hybridization-competition method to isolate *EuNOD-CHI* from a root nodule cDNA library of *Elaeagnus umbellata*. This cDNA clone encodes chalcone isomerase (CHI) for a protein of 256 amino-acid residues and a mature molecular mass of 28 kDa. Multiple sequence alignment and phylogenetic analysis have demonstrated that EuNOD-CHI can be classified as Type I. Moreover, northern hybridization shows that the *EuNOD-CHI* gene is highly expressed in root nodules, with levels increasing during nodule development. The highest level of expression is at 6 to 8 weeks after inoculation, decreasing thereafter. Genomic Southern hybridization also demonstrates that *EuNOD-CHI* has as many as two copies in the *E. umbellata* genome. Taken together with the previous results, we propose that the higher expression level of the *EuNOD-CHI* gene in root nodules is likely associated with this species' defense mechanism against infection by *Frankia*.**

*Keywords*: cDNA, chalcone isomerase, *Elaeagnus umbellata*, *Frankia*, root nodule

Symbiosis between the actinomycete *Frankia* and plant roots enables actinorhizal root nodules to fix atmospheric nitrogen in dicotyledonous plants belonging to eight families and 25 genera. These nodules are specialized structures comprising modified lateral roots that originate from the pericycle and possess a central vascular system (Berry and Sunell, 1990). In contrast, leguminous root nodules resemble shoots, having a peripheral vascular bundle and originating from the root cortex (Hirsch, 1992). Due to activity in the apical meristem, those nodule lobes show an indeterminate growth pattern and have developmental zonation (Ribeiro et al., 1995).

In legume-rhizobium symbiosis, flavonoids play a critical function as inducers of nodulation (*nod*) genes. These genes are involved in the biosynthesis of bacterial signaling molecules, i.e., Nod-factors. Flavonoids, which are products of the phenylpropanoid pathway, interact with a transcriptional activator, NodD, to turn on the transcription of bacterial *nod* genes (Crawford et al., 2000). Other postulated roles for flavonoids include the chemo-attraction of rhizobia to roots, as well as enhancing the growth of rhizobia cultured in minimal media (Caetano-Anollés et al., 1988; Hartwig et al., 1991).

Chalcone isomerase (CHI, EC 5.5.1.6) is involved in very early step of the flavonoid biosynthetic pathway. CHI mediates right next step of chalcone synthase (CHS) and catalyzes the stereo-specific isomerization of chalcones into their corresponding (2S)-flavanones, e.g., naringenin and liquiritigenin. Using X-ray crystallography, Jez et al. (2000) have elucidated the protein structure for the CHI enzyme. In most plant species, CHI is encoded by only one or two genes, while CHS by multiple genes (see review by Holton and Cornish, 1995). On the other hand, four CHI genes have been isolated from *Lotus japonicus* (Shimada et al., 2003).

Genes involved in the phenylpropanoid pathway have been identified in several legume species, and their expression patterns have been examined in infected roots and root nodules (Yang et al., 1992; McKhann et al., 1997; Goormachtig et al., 1999). However, only limited research has been conducted on the phenylpropanoid biosynthetic genes in actinorhizal plants that interact with *Frankia*. For example, a cDNA clone encoding CHS has been isolated and analyzed from the root nodules of *Casuarina glauca* (Laplaze et al., 1999). Likewise, since the time that a cDNA clone encoding cysteine proteinase was first isolated from the actinorhizal plant, *Alnus glutinosa* (Goetting-Minesky and Mullin, 1994), root nodule-specific or -enhanced genes have been identified from *Alnus* and *Casuarina* (see reviews by Pawlowski, 1997; Wall, 2000).

*Alnus* differs from *Elaeagnus* in both its assimilation pathway for fixed nitrogen and in its infection pathway (Schubert, 1986; Berry and Sunell, 1990). Therefore, to better understand root nodule development in actinorhizal plants, we previously isolated several nodule-specific or -enhanced cDNA clones from *E. umbellata*, using the hybridization-competition method (Kim et al., 1999; Kim and An, 1999, 2002; Lee et al., 2001). In the current study, our objective was to identify and

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characterize a cDNA clone that encodes CHI from a root nodule cDNA library for *E. umbellata*. In particular, we wished to determine how the CHI gene is involved in the phenylpropanoid biosynthetic pathway for an actinorhizal plant interacting with *Frankia*.

## MATERIALS AND METHODS

### Bacterial Strain and Plant Material

To nodulate seedlings of *E. umbellata*, we used *Frankia* strain EuIK1, which was isolated from *Elaeagnus* root nodules and maintained in pure culture in a DPM medium without a nitrogen source (Kim et al., 1993). RNA for the construction of the nodule cDNA library was isolated at various developmental stages from 6-month-old *Frankia*-inoculated plants. Seedlings that were not inoculated were used for isolating total RNA from the leaves and roots. To analyze changes in gene expression during root nodule development, nodules were harvested at 4, 6, 8, and 10 weeks after inoculation (WAI). The harvested tissues were frozen in liquid nitrogen and stored at −80°C.

### Isolation of Nucleic Acids

Total RNA and genomic DNA were isolated as described by Doyle and Doyle (1990), with the following modification: PVPP was added during grinding with liquid nitrogen to remove phenolic compounds. RNase-free DNase (Promega, USA) was used to remove genomic DNA contamination in the RNA samples during RNA purification.

### cDNA Library Screening

Construction and screening of a root nodule cDNA library was described previously by Kim (1998). Nodule-specific or -enhanced cDNA clones were isolated from $1\times10^5$ of phage clones according to the hybridization-competition method of Mangiarotti et al. (1981).

### Cloning and Sequence Analysis

Positive phage clones from the primary and secondary differential screening were changed into phagemid clones according to the manufacturer's *in-vivo* excision protocol (Stratagene, USA). Phagemid DNA was deleted unidirectionally with exonuclease III and S1 nuclease, using a double-stranded Nested Deletion Kit (Pharmacia Biotech, Sweden), as based on the protocol of Henikoff (1984). The nucleotide sequences were determined via the dideoxynucleotide chain termination method (Sanger et al., 1977), using *Taq* polymerase (Promega). Sequences were analyzed with the ExPASy Molecular Biology Server (URL http://kr.expasy.org) and the BLAST program (Altschul et al., 1990). A phylogenetic tree was generated using the PHYLIP program.

### DNA and RNA Gel Blot Analysis

For the DNA analysis, the genomic DNA (10 μg) purified from the leaves of *E. umbellata* was digested with restriction enzymes, separated it on a 1% agarose gel, and then transferred to a nylon membrane (Amersham, UK) by the capillary blotting method (Sambrook et al., 1989). For RNA analysis, total RNA (20 μg) was separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. The DNA blot was hybridized overnight with a $^{32}$P-labeled DNA probe under the following conditions: 6 X SSC [0.9 M NaCl and 0.09 M sodium citrate (pH 7.0)], 5 X Denhardt's solution, and 0.1% SDS, at 57°C. The hybridized blots were washed at 57°C, with the salt concentration gradually decreasing to 1 X SSC, and exposed to X-ray film (Fuji, Japan). For RNA blot, hybridization and washing were carried out at 63°C.

### Reverse Transcription-PCR

RT-PCR was done to analyze expression patterns during root nodule development. PCR primers were designed to specifically amplify the 3′ UTR of the *EuNOD-CHI* gene. The primer sequences were, for CHIRTF, 5′-GGA-AGTTGAAGTGTAAGCAT-3′; and for CHIRTR, 5′-TGGG-GCAAAGCATTAAACTA-3′. Total RNA (1 μg) was used as template for reverse transcription after treatment with RNase-free DNase (Promega). The PCR cycles included 95°C for 5 min of initial denaturation, followed by 94°C for 15 sec, 55°C (67°C for *nif*H) for 30 sec, and 72°C for 45 sec (for a total of 26 cycles), followed by a 5-min final extension at 72°C. The amplified PCR products were separated on a 1.2% agarose gel, transferred to a nylon membrane, and probed with $^{32}$P-labeled inserts of *EuNOD-CHI* and *nif*H (Kim and An, 1997).

## RESULTS

### Isolation and Characterization of the cDNA Clone Encoding Chalcone Isomerase

We have isolated a cDNA clone showing high

sequence homology with the previously reported chalcone isomerase from a root nodule cDNA library of *E. umbellata*. Our hybridization-competition method involved a mixture of radio-labeled single-strand root nodule cDNA probe and an excess of total RNAs from uninfected roots and leaves (Kim, 1998). The full-length sequence of this cDNA clone, named *EuNOD-CHI* (*E. umbellata* Nodule Chalcone Isomerase), has been determined and analyzed.

The CHI clone, EuNOD-CHI, encodes a protein for 256 amino-acid residues, with a molecular weight of 28 kDa and a calculated pI value of 4.99. No organellar targeting sequences were found in the sequences, indicating that EuNOD-CHI might be a cytosolic enzyme. CHIs are classified into two types (Kimura et al., 2001; Shimada et al., 2003), with each predicted to have different substrate preferences, that is, 6'-hydroxychalcone for Type-I CHIs and 6'-deoxychalcone for

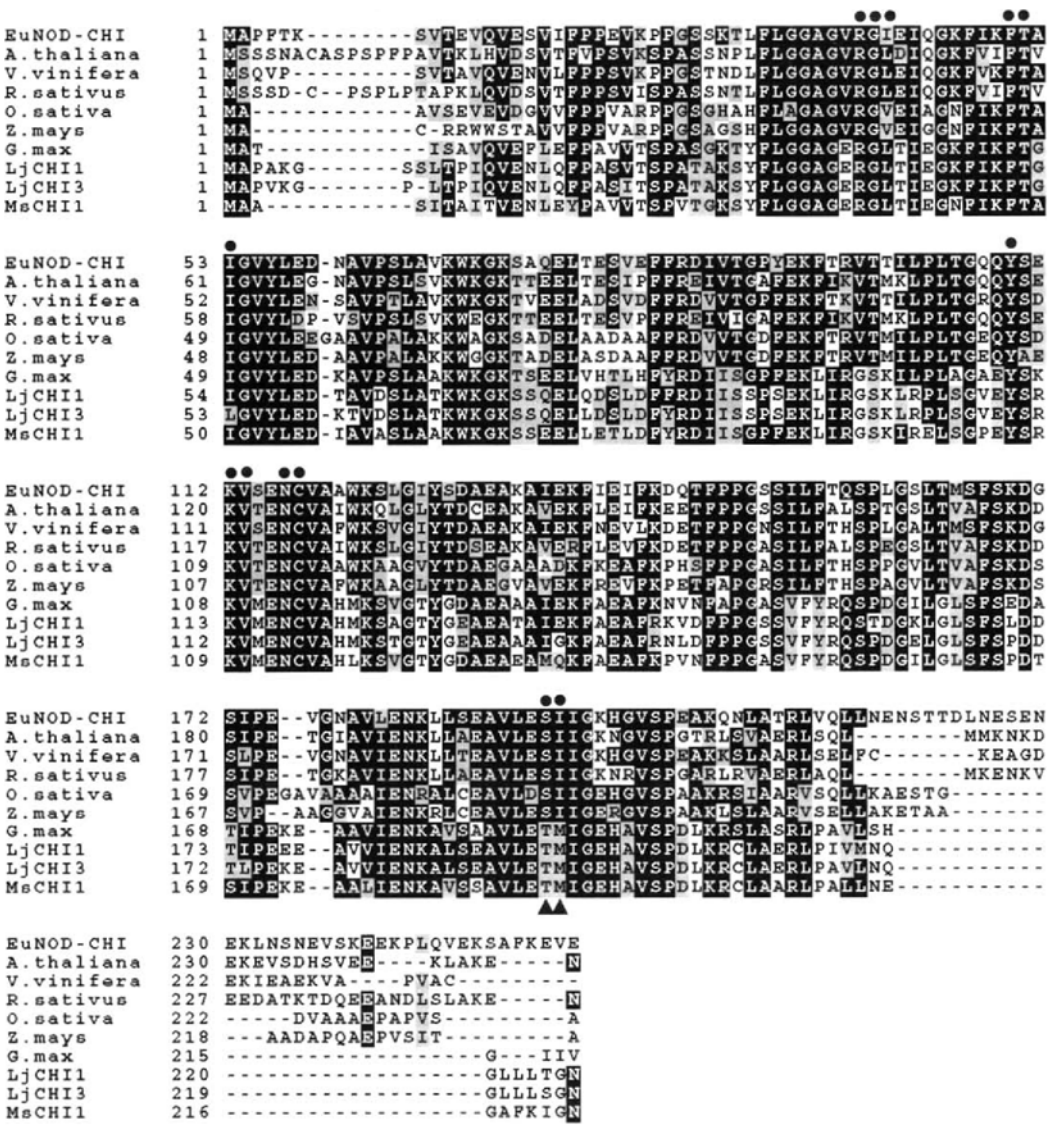


**Figure 1.** Multiple sequence alignment of EuNOD-CHI and previously reported CHIs using CLUSTAL W. The amino-acid residues that form the active site are indicated with filled circles. Arrow heads indicate the residues proposed to affect substrate preference between 6'-deoxychalcone and 6'-hydroxychalcone. LjCHI1, *Lotus japonicus* CHI1; LjCHI2, *Lotus japonicus*; MsCHI1, *Medicago sativa* CHI1. Sources and GenBank accession numbers of CHI sequences used in this alignment are EuNOD-CHI (in this study, AF061808), *Arabidopsis thaliana* (P41088), *Raphanus sativus* (AAB87071), *Oryza sativa* (AAO65886), *Zea mays* (CAA80441), *Glycine max* (AAK69432), *Lotus japonicus* CHI1 (AB054801), *Lotus japonicus* CHI3 (AB073787), and *Medicago sativa* CHI1 (M91079).

Type-II CHIs (Jez et al., 2000). The deduced amino acid sequences within the same type share high sequence identity (>70%), whereas the similarity between Types I and II is <55%. In our study, EuNOD-CHI showed identities of 62% and 77% (maize and grape, respectively), both species possessing Type-I CHIs. In contrast, similarities with Type-II CHIs were 52% (pea) and 59% (soybean). This suggests that EuNOD-CHI could be classified as being of Type I.

We performed multiple sequence alignment of EuNOD-CHI and other previously reported CHIs (Fig. 1). CHIs are highly conserved, even between the two types, except for the N- and C-termini. The residues forming the active site are also conserved, e.g., Arg-39, Gly-40, Phe-50, Thr-51, Ile-53, Tyr-109, Lys-112, Val-113, Asn-116, and Cys-117 (Jez et al., 2000). Leu-41 conserved in most CHIs was substituted by isoleucine in *E. umbellata* and by valine in the monocots, rice and maize (Fig. 1). The two residues in the C-terminus region, Thr-190 and Met-191 in alfalfa, which presumably determine substrate preference for Type-II CHIs, were strictly conserved in all the Type-II CHIs investigated here (Fig. 1). In contrast, the two amino-acid residues were serine and isoleucine in Type-I CHIs, including *E. umbellata* (Ser-193 and Ile-194) (Fig. 1).

Phylogenetic analysis also demonstrated that EuNOD-CHI can be grouped with the Type-I CHIs from non-leguminous plants, which supports our results from the multiple sequence alignment and sequence identity. In contrast, the Type-II CHIs from leguminous plants were clustered into a different group (Fig. 2).

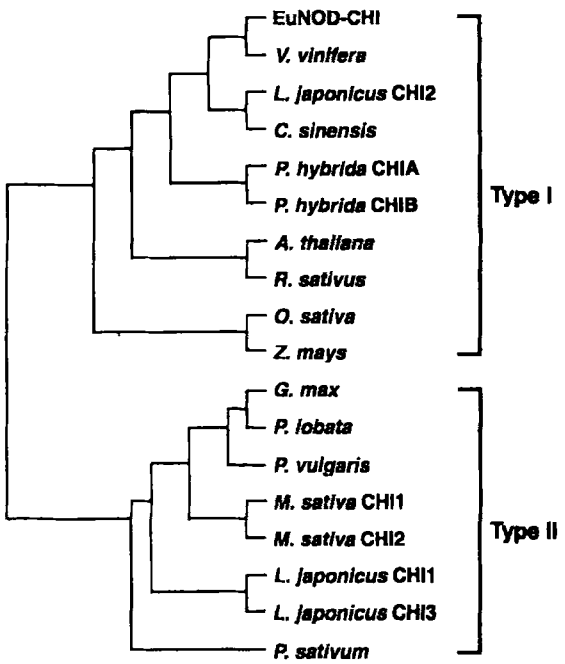


**Figure 2.** Phylogenetic tree based on deduced amino acid sequences of various CHIs. Amino acid sequences were aligned using the CLUSTAL W program and a UPGMA tree was generated using the PHYLIP program. EuNOD-CHI was classified into the Type-I CHI group with CHIs from non-leguminous plants. Sources and GenBank accession numbers of CHI sequences used in this alignment are EuNOD-CHI (in this study, AF061808), *Vitis vinifera* (P51117), *Lotus japonicus* CHI2 (AB054802), *Citrus sinensis* (BAA36552), *Petunia hybrida* CHIA (AAF60296), *Petunia hybrida* CHIB (P11651), *Arabidopsis thaliana* (P41088), *Raphanus sativus* (AAB87071), *Oryza sativa* (AAO65886), *Zea mays* (CAA80441), *Glycine max* (AAK69432), *Pueraria lobata* (Q43056), *Phaseolus vulgaris* (P14298), *Medicago sativa* CHI1 (M91079), *Medicago sativa* CHI2 (M91080), *Lotus japonicus* CHI1 (AB054801), *Lotus japonicus* CHI3 (AB073787), and *Pisum sativum* (AAA50174 ).

## Expression Pattern of *EuNOD-CHI*

Genes that encode flavonoid-biosynthetic enzymes such as CHS and chalcone reductase (CHR) are highly expressed in the root nodules of leguminous plants (Yang et al., 1992; Goormachtig et al., 1999). Here, the clone encoding EuNOD-CHI was isolated from a root nodule cDNA library using the hybridization-competition method. We hypothesized that this gene would have a nodule-specific or -enhanced expression pattern. Therefore, we purified total RNA from young and mature leaves, uninoculated roots, and root nodules, and subjected these to northern hybridization using the full-length insert of the *EuNOD-CHI* clone as a probe. Transcripts for EuNOD-CHI were detected at very high levels in root nodules compared with the lesser amounts found in uninoculated roots. The *EuNOD-CHI* gene was also more strongly expressed in young rather than mature leaves (Fig. 3A).

We used RT-PCR to examine *EuNOD-CHI* expression patterns during nodule development. First, *nif*H transcripts that encode nitrogenase reductase, a component of the nitrogenase enzyme complex, were amplified as a positive control and molecular marker for root nodule development. *nif*H showed the highest expression level at 6 to 8 weeks after inoculation (WAI), decreasing thereafter (Fig. 3B). *EuNOD-CHI* also manifested a similar expression pattern (Fig. 3B). Using the same RT-product, we also analyzed the activity of a polyubiquitin gene (Kim and An, 1999), and found that it was highly expressed in the root nodule, especially at 4 and 10 WAI. This indicates that the starting amount of RNA used in the RT reaction was equivalent at each developmental stage.

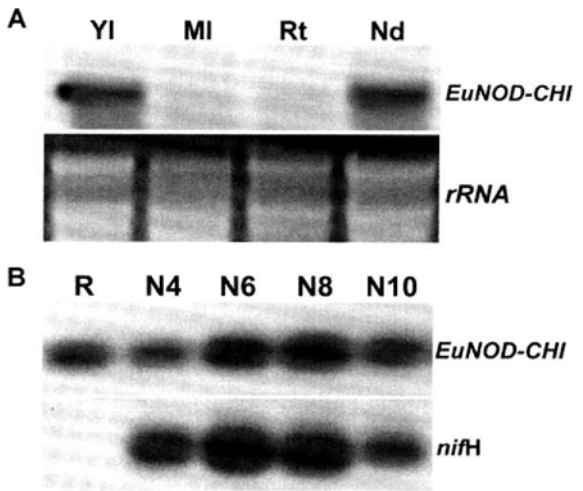


**Figure 3.** Expression pattern of *EuNOD-CHI* gene. **A,** Northern hybridization analysis for different tissues. Yl, young leaves; Ml, mature leaves; Rt, uninoculated roots; Nd, root nodule. **B,** RT-PCR analysis of *EuNOD-CHI* expression during root nodule development. *nif*H transcripts were amplified as a marker gene. R, uninoculated roots; N4, nodule at 4 WAI (weeks after inoculation); N6, nodule at 6 WAI; N8, nodule at 8 WAI; N10, nodule at 10 WAI.

### *EuNOD-CHI* Genes in the Genome of *E. umbellata*

Chalcone isomerase is encoded by one or more genes, depending on the plant species (McKhann and Hirsch, 1994; Sparvoli et al., 1994; Song et al., 1998). Recently, four CHI genes have been identified from a model legume, *Lotus japonicus* (Shimada et al., 2003). Therefore, to determine the number of *EuNOD-CHI* genes in the *E. umbellata* genome, we performed genomic Southern hybridization using the full-length insert of *EuNOD-CHI* clone as a probe under low stringency (Fig. 4). Total genomic DNA was digested with three restriction enzymes, *Eco*RI, *Hin*dIII, and *Xba*I, which do not cut the cDNA clone. As shown in Figure 4, two or three strong hybridizing signals were detected in each lane; 9.6-, 5.0-, and 3.5-kb *Eco*RI fragments, 2.8- and 2.7-kb *Hin*dIII fragments, and 9.6-, 6.5-, and 5.0-kb *Xba*I fragments. Therefore, this hybridization pattern suggests that *EuNOD-CHI* may be encoded by 2 copy genes.

## DISCUSSION

Flavonoids are plant-specific secondary metabolites derived from the phenylpropanoid pathway. They are involved in various biological processes, including flower pigmentation, protection against UV irradiation, and defense against microbial pathogens. Flavonoids also play key roles in the interaction between plants and microbes (Winkel-Shirley, 2001). In particular, leguminous species produce flavonoids and isoflavonoids that are essential for legume-rhizobia interactions.

Chalcone isomerase is an important enzyme in the phenylpropanoid pathway for producing various (iso) flavonoids. CHIs are classified into two types (Kimura et al., 2001; Shimada et al., 2003). Those of Type I generally are found in non-leguminous species, and isomerize only 6'-hydroxychalcone to produce (2S)-naringenin (5-hydroxyflavanone). In contrast, the Type-II CHIs, from leguminous plants, convert both 6'-deoxychalcone and 6'-hydroxychalcone to (2S)-liquiritigenin (5-deoxyflavanone) and (2S)-naringenin, respectively. The latter is further metabolized to produce general 5-hydroxyflavonoids. In contrast, (2S)-liquiritigenin is further metabolized to form 5-deoxy(iso)flavonoids, i.e., legume-specific flavonoids. Our multiple sequence alignment and phylogenetic analysis (Fig. 1, 2) suggest that EuNOD-CHI can be classified as a Type-I CHI. This would indicate that it is probably involved in producing general 5-hydroxyflavonoids, such as anthocyanin and flavonol, rather than 5-deoxy(iso)flavonoids.

Although actinorhizal plants, including *E. umbellata*, are non-leguminous species, they can nodulate through interaction with symbiotic microbes such as *Frankia*. No clear evidence is yet available for actinorhizal symbiosis that would demonstrate whether flavonoids play similar signaling roles as for legume-*Rhizobium* symbiosis. Flavonoid-like compounds and flavonols reportedly have stimulatory or inhibitory effects on the nodulation of *Alnus* species by *Frankia* (Benoit and Berry, 1997; Hughes et al., 1999). It would be very interesting to determine whether Type-II CHIs are present in the genome of *E. umbellata* and other actinorhizal plants, such as *Alnus*, *Myrica*, and *Casuarina*.

Our data for genomic Southern hybridization under low stringency showed that probably two genes encode CHI homologues in the *E. umbellata* genome (Fig. 4). Therefore, if we could isolate the Type-II CHI genes in actinorhizal plants, this would provide plausible evidence that both CHI types produce a broad range of flavonoids, including 5-hydroxyflavonoids and 5-deoxy (iso)flavonoids, that can serve as signaling compounds for efficient nodulation in actinorhizal species. Unfortunately, *nod* genes have not yet been isolated from *Frankia* strains. However, the presence of a root hair-deforming factor has been recognized in the actinorhizal plant-*Frankia* system (van Ghelue et al., 1997). However, this factor is genetically and biochemically divergent from the rhizobial Nod-factor (Cérémonie et al., 1998, 1999).

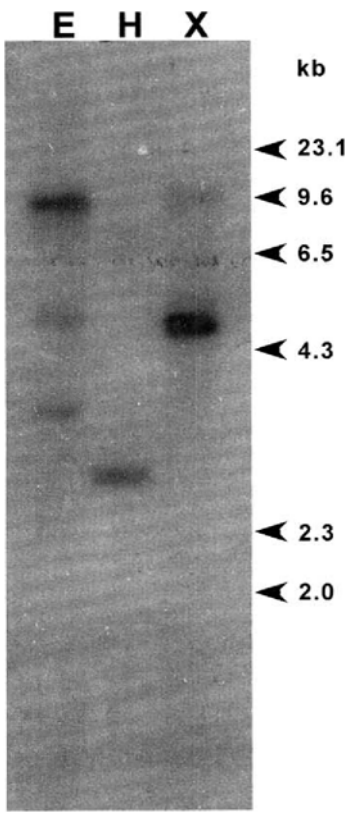


**Figure 4.** Genomic Southern analysis for *EuNOD-CHI* gene. DNA blot was probed with $^{32}$P-labeled full-length *EuNOD-CHI* clone and washed under low-stringency conditions. E, *Eco*RI; H, *Hin*dIII; X, *Xba*I.

The phenylpropanoid biosynthetic genes are up-regulated by various environmental stresses, including pathogenic microbes. With their many analogies between pathogenesis and symbiosis, symbiotic bacteria are considered similar to parasites. Many defense-related genes have been isolated from the early and late stages of nodulation (see review by Baron and Zambryski, 1995). Based on these observations, the accumulation of *EuNOD-CHI* transcripts found in our root nodules might mimic a defense response against infection by *Frankia* (Fig. 3A and B).

The expression pattern of *EuNOD-CHI* during nodule development is similar to that of chitinase genes, which have also been isolated from *Elaeagnus* root nodules (Kim and An, 2002). Laplaze et al. (1999) have detected high levels of CHS transcripts in the uninfected cortex cells of *Casuarina glauca*. These cells contain the flavan class of flavonoids (derived from the phenylpropanoid pathway) in the root nodules, which suggests that those uninfected cells delimit *Frankia*-infected compartments. Moreover, in the root nodules induced by rhizobia in *Sesbania rostrata*, expression of the CHR gene is correlated with both nodule development and bacterial invasion. CHR transcripts were detected in the uninfected cells of the central tissue that contains rhizobial cells (Goormachtig et al., 1999). Based on those previous reports, we predict that *EuNOD-CHI*, classified as a Type-I CHI, is expressed in the uninfected cells of the root nodule.

In conclusion, we report here the identification of a Type-I *CHI* clone that is highly expressed in the root nodules of an actinorhizal species, *E. umbellata*. The clone, *EuNOD-CHI*, is probably involved in the production of 5-hydroxyflavonoids rather than 5-deoxy (iso)flavonoids. This clone provides a molecular tool for elucidating the role of flavonoids (or isoflavonoids) as defense chemicals or signaling molecules in actinorhizal symbiosis. Therefore, to better understand the functional roles of CHI proteins in the actinorhizal plant, we are conducting *in-situ* localization analysis of *EuNOD-CHI* transcripts in root nodules, as well as the cloning of Type-II CHIs.

## ACKNOWLEDGMENTS

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