Differential Expression Patterns of an Acidic Chitinase and a Basic Chitinase in the Root Nodule of *Elaeagnus umbellata*

Ho Bang Kim and Chung Sun An

School of Biological Sciences, Seoul National University, Seoul 151-742, Korea

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Two cDNA clones encoding chitinase were isolated from a root nodule cDNA library of *Elaeagnus umbellata* by the hybridization-competition method. The two clones, EuNOD-CHT1 and EuNOD-CHT2, encode for 335 and 317 amino acid residues with the molecular mass of mature proteins being 33.3 and 31.1 kDa, respectively. The two chitinases showed similar protein structures consisting of four domains: hydrophobic signal peptide domain, cysteine-rich chitin-binding domain, hinge domain, and catalytic domain. The EuNOD-CHT1 gene showed similar expression levels in root nodules and leaves, with no detection of transcripts in the roots. The EuNOD-CHT2 gene was expressed at similarly high levels in the roots and root nodules, but at a very low level in the leaves. In situ hybridization showed that EuNOD-CHT1 transcripts were strongly detected in the meristem zone, but weakly detected in the outer cortex layer of the root nodule and in the uninfected cells of the fixation zone. On the other hand, EuNOD-CHT2 transcripts were strongly detected in the infected cells of the fixation zone and central vascular system, but weakly detected in the senescence zone. Our results suggest that the two chitinases may play different biological roles in the root nodule. EuNOD-CHT2 may be involved in a defense response against internal symbionts, external pathogens, or both, while EuNOD-CHT1 may be involved in normal plant development as well as in a defensive role against external pathogens.

Additional keywords: actinorhizal plant, *Frankia*.

Actinorhizal root nodules are able to fix atmospheric nitrogen owing to the symbiosis between the members of the actinomycete genus *Frankia* and the roots of dicotyledonous plants that belong to eight plant families and 25 genera. They resemble lateral roots with a central vascular system and originate from pericycle (Berry and Sunell 1990), while leguminous root nodules resemble shoots with a peripheral vascular bundle and originate from the root cortex (Hirsch 1992). Because of the presence of apical meristem activity, its nodule lobes show an indeterminate growth pattern and, thus, consist of four different zones: meristem zone, prefixation zone, fixation zone, and senescence zone (Ribeiro et al. 1995).

Corresponding author: Chung Sun An; Telephone: 2-880-6678; Fax: 2-872-6881; E-mail: ancs@plaza.snu.ac.kr

Complete nucleotide sequence data of EuNOD-CHT1 and EuNOD-CHT2 are available in the GenBank database under the accession numbers AF061805 and AF061806, respectively.

Chitinase (EC 3.2.1.14) catalyzes the hydrolysis of chitin, a linear homopolymer of β -1,4-linked *N*-acetylglucosamine (GlcNAc) residues, and is an abundant protein found in a wide variety of seed-producing plants (Collinge et al. 1993; Graham and Sticklen 1994). Plant chitinases have been best characterized as tools in the defense response against pathogenic microbes. Chitinase can induce defense responses by generating elicitors and can inhibit the growth of many fungal pathogens by causing lysis of hyphal tips (Collinge et al. 1993). In addition, there are several reports that plant chitinases might be involved in normal plant growth and development, such as the rescue of a carrot somatic embryo mutant by a purified acidic chitinase (de Jong et al. 1992), the increased expression level of chitinase during normal plant development (Harikrishna et al. 1996; Leung 1992; Neale et al. 1990; Takakura et al. 2000), and the appearance of chitinases during leaf senescence (Hanfrey et al. 1996).

Chitinases are thought to be involved in nodulation. Vasse and colleagues (1993) showed that chitinases might play a role as part of an autoregulation mechanism of the plant to control infection and nodulation by aborting the infection thread once the first nodule primordia are induced in the alfalfa-*Rhizobium meliloti* interaction. In soybean nodules induced by the wild-type *Bradyrhizobium japonicum* strain or a mutant strain eliciting a hypersensitive response, it was suggested that chitinases may be involved in the protection of nodule tissues against external pathogens (Staehelin et al. 1992). Finally, there are several reports that nodulation might be controlled at the initial step through the degradation of lipochitooligosaccharide (LCO) nodulation signals by chitinases secreted from the root tissue of a host plant (Staehelin et al. 1994, 1995).

Up to now, studies on nodule-specific or enhanced genes have concentrated on several leguminous plants. However, after initial isolation of a cDNA clone encoding cysteine proteinase from the actinorhizal plant *Alnus glutinosa* (Goetting-Minesky and Mullin 1994), several actinorhizal nodule-specific or enhanced genes have been isolated from *Alnus* and *Casuarina* species (Laplaze et al. 2000; Pawlowski et al. 1997; Ribeiro et al. 1995). *Alnus* species are known to be different from *Elaeagnus* species in the assimilation pathway of fixed nitrogen and the infection pathway (Berry and Sunell 1990; Schubert 1986). To better understand nodule development in actinorhizal plants, we isolated several nodule-specific or enhanced cDNA clones from an *Elaeagnus* species by the hybridization-competition method (Kim and An 1999; Kim et al. 1999).

In this study, we have isolated and characterized two cDNA clones encoding chitinase from a root nodule cDNA library of *E. umbellata*. We also have analyzed their expression patterns by Northern hybridization, reverse-transcription-polymerase

chain reaction (RT-PCR), and in situ hybridization to better understand the biological roles of the chitinase genes in the root nodule. From these results, we will discuss putative biological roles of two chitinase genes in the actinorhizal nodule.

RESULTS

Molecular characterization of two chitinases, EuNOD-CHT1 and EuNOD-CHT2.

Two different chitinase clones, EuNOD-CHT1 and EuNOD-CHT2 (*E. umbellata* nodule chitinase), were isolated from a root nodule cDNA library by the hybridization-competition method using a radiolabeled, single-strand root nodule cDNA

probe and an excess of total RNAs from the roots and leaves (Kim 1998). The two chitinase clones encode an acidic chitinase and a basic chitinase; the calculated molecular masses of mature proteins are 33.3 and 31.1 kDa and the calculated pI values are 4.28 and 8.26, respectively.

Figure 1 shows the deduced amino acid sequences from EuNOD-CHT1 and EuNOD-CHT2 aligned with previously reported class I chitinases. The two chitinases each consist of four domains: hydrophobic signal peptide domain, cysteine-rich chitin-binding domain, hinge domain, and catalytic domain. Eight cysteine residues, which are known to play an important role in chitin binding, are well conserved among class I chitinases (Fig. 1). The hinge domain of EuNOD-CHT1 was

Fig. 1. Amino acid multiple sequence alignment of EuNOD-CHT1 and EuNOD-CHT2 with previously reported class I chitinases using Clustal W. Black shading indicates perfectly conserved amino acid residues among all sequences. Open circles are eight conserved cysteine residues involved in chitin binding. The filled triangles mean relatively conserved amino acid residues of active sites in the catalytic domain of chitinase. The bold italic residues in the C terminal of EuNOD-CHT2 are putative vacuole-targeting sequences, which are not present on the mature protein. The two potential *N*-glycosylation sites on EuNOD-CHT2 are underlined. The sources of chitinase sequences used in this alignment are EuNOD-CHT1 (this work; GenBank accession no. AF061805), EuNOD-CHT2 (this work; GenBank accession no. AF061806), bean (GenBank accession no. M13968), alfalfa (GenBank accession no. U83591), pea (GenBank accession no. X63899), tobacco (GenBank accession no. X16939), grape (GenBank accession no. Z54234), maize (GenBank accession no. L00973), and rice (GenBank accession no. X87109).

longer than that of other chitinases and had a stretch of 15 hydrophobic glycine residues. This domain is known to vary in its size, is rich in glycine and proline, and is unconserved among plant chitinases (Payne et al. 1990; Shinshi et al. 1990). There is a well-conserved sequence motif in the catalytic domain of chitinases, GP-X-Q-X-S-X-NYNYG-XX-Q, which is known to be the active site (Fig. 1). The underlined tyrosine, known to be essential for the active site in a chitinase from maize, was also found in β -galactosidase, which acts on β -1,4-linked saccharides (Verburg et al. 1992). EuNOD-CHT2 includes two potential *N*-linked glycosylation sites, N-X-S/T (Bause 1983) in the amino acid sequence N-P-S, and N-P-T in the middle of the catalytic domain (Fig. 1). The amino acid se-

quences of EuNOD-CHT1 and EuNOD-CHT2 showed more than 60% identity with those of class I and class II plant chitinases and also approximately 40% and below 30% identity with those of class IV and class III chitinases, respectively (data not shown).

Expression patterns in different tissues.

The expression patterns of EuNOD-CHT1 and EuNOD-CHT2 among the leaves, roots, and root nodules were studied by RNA gel blot analysis (Fig. 2). As shown in Figure 2, EuNOD-CHT1 transcripts were detected at similar levels in the root nodules and the leaves but were not detected in the roots. On the other hand, EuNOD-CHT2 expression was detected in all tissues, although the gene was expressed at about five-times-higher levels in the roots and root nodules compared with the leaves.

In situ localization in the root nodule.

The localization of transcripts of EuNOD-CHT1 and EuNOD-CHT2 in the root nodule was determined by in situ hybridization for longitudinal and transverse sections with digoxigenin (DIG)-labeled antisense and sense RNA probes, respectively (Figs. 3 and 4). EuNOD-CHT1 transcripts were strongly detected in the meristem zone, which lies in the distal part of the root nodule and consists of small-size cells (Fig. 3A and B). The transcripts also were very weakly detected in the outer cortex layer, including the periderm, and in the uninfected cells of the fixation zone (Fig. 3C and D). No hybridization signals were detected in the root nodule sections hybridized with the sense probe (Fig. 3E to H). EuNOD-CHT2 transcripts were strongly detected in the infected and nitrogen-fixing cells of the fixation zone (Fig. 4A and B), where the *nifH* marker gene was very strongly expressed (data not shown), and in the central vascular system (Fig. 4A, B, and D), and weakly detected in the senescence zone (Fig. 4C). In our previous results (Kim

Fig. 2. Expression patterns of chitinase genes among different organs. Northern blots containing 20 μ g of total RNA per lane were hybridized with the 32 P-labeled full insert of pEuNOD-CHT1 and pEuNOD-CHT2. RNA gel was stained with ethidium bromide to check the quantity and the quality of RNA before transfer to nylon membrane. L = leaf, R = root, and N = root nodule.

Fig. 3. In situ localization of EuNOD-CHT1 transcripts in the root nodule. **A**, A longitudinal section hybridized with an antisense probe; **B**, detailed view of the meristem zone of **A**; **C**, detailed view of the outer cortex layer, including periderm, of **A**; **D**, detailed view of the fixation zone of **A**; **E**, a longitudinal section hybridized with a sense probe; **F**, detailed view of the meristem zone of **E**; **G**, detailed view of the outer cortex layer, including periderm, of **E**; and **H**, detailed view of the fixation zone of **E**. MZ = meristem zone, FZ = fixation zone, PD = periderm, VS = vascular system, UC = uninfected cells, and IC = infected cells. **A** and **E**, Bars = 250 μ m. **B** to **D** and **F** to **H**, Bars = 50 μ m.

and An 1999; Kim et al. 1999), the *nifH* gene encoding nitrogenase reductase was used as a marker gene to identify the infected and nitrogen-fixing cells from the uninfected cells of the fixation zone. No hybridization signals were detected in the root nodule sections hybridized with the sense probe (Fig. 4E to H).

Expression patterns in root segments.

To analyze gene expression patterns of EuNOD-CHT1 and EuNOD-CHT2 in different root zones, the root was roughly divided into three parts: root tips containing root meristem, elongation zone, and maturation zone containing secondary roots. Total RNA was isolated from these root segments and subjected to RT-PCR analysis. EuNOD-CHT1 transcripts were detected only in the root tips and the maturation zone containing secondary roots, but not in the elongation zone (Fig. 5). On the other hand, EuNOD-CHT2 transcripts were detected in all segments (Fig. 5).

DISCUSSION

We have cloned two different plant chitinases from a root nodule cDNA library of an actinorhizal species, *E. umbellata*, and analyzed their expression patterns to understand their biological roles in the root nodule. Plant chitinases are divided into six classes based on their amino acid sequence features, and class I chitinases are divided into two subclasses (Ia and Ib) depending on the presence of C-terminal extension for vacuole targeting (Collinge et al. 1993; Neuhaus et al. 1996). EuNOD-CHT2 has a C-terminal extension motif (G-S-G-L-L-V-D-T-M) (Fig. 1) with high hydrophobicity similar to that of tobacco chitinase, which is necessary and sufficient to target the tobacco protein to the plant vacuole (Neuhaus et al. 1991). Therefore, EuNOD-CHT2 may be localized in the vacuole also.

EuNOD-CHT1 does not have a C-terminal extension similar to that of the tobacco basic chitinase, although the C-terminal part of EuNOD-CHT1 contains several hydrophobic amino acid residues. The prediction of protein localization sites using the PSORT program (Human Genome Center, Institute of Medical Science, University of Tokyo, Japan) showed that EuNOD-CHT1 might be secreted into the apoplastic space after synthesis and modification of the protein, like many other acidic or basic chitinases. According to the amino acid sequence features of the two chitinases and the plant chitinase classification rule, EuNOD-CHT1 and EuNOD-CHT2 can be classified into class Ib and class Ia, respectively.

Chitinases known as pathogenesis-related (PR) proteins were induced not only by biotic factors such as fungal pathogens and bacterial infection but also by abiotic factors such as wounding and heavy metals or by stress-related plant hormones such as ethylene, salicylic acid, and jasmonic acid (Collinge et al. 1993; Graham and Sticklen 1994). EuNOD-CHT1 transcripts were highly detected in the root nodules (Fig. 2) and weakly detected in the periderm and the uninfected cells in the outer cortex (Fig. 3A and C), suggesting a defensive role in the root nodule. By many analogies between pathogenesis and symbiosis, symbiotic bacteria have also been considered as being similar to parasites (Baron and Zambryski 1995; Vance 1983). From this viewpoint, chitinase induction during nodule organogenesis has been considered a defenselike response against infecting symbionts to control nodulation (Vasse et al. 1993). EuNOD-CHT1, however, may be involved in protecting nodule tissues, especially the root nodule meristem, against external pathogenic bacteria or incompatible symbionts rather than a defense response against internal pathogenic symbionts. This hypothesis can be supported by the results that EuNOD-CHT1 transcripts are hardly detected in the prefixation zone and the fixation zone, regardless of infection, but are mostly detected

Fig. 4. In situ localization of EuNOD-CHT2 transcripts in the root nodule. **A**, A longitudinal section hybridized with an antisense probe; **B**, detailed view of the fixation zone of **A**; **C**, detailed view of the senescence zone of **A**; **D**, a transverse section hybridized with an antisense probe; **E**, a longitudinal section hybridized with a sense probe; **F**, detailed view of the fixation zone of **E**; **G**, detailed view of the senescence zone of **E**; and **H**, a transverse section hybridized with a sense probe. MZ = meristem zone, FZ = fixation zone, SZ = senescence zone, PD = periderm, VS = vascular system, UC = uninfected cells, and IC = infected cells. **A** and **E**, Bars = 250 µm. **B** to **D** and **F** to **H**, Bars = 50 µm.

in the meristem zone and the uninfected outer cortex layer (Fig. 3A to D). This hypothesis also can be supported from previous results that chitinase activities were detected primarily in the cortex of an effective nitrogen-fixing soybean nodule, while they were strongly enhanced in the central infected zone as well as in the cortex of an ineffective (fix⁻) nodule (Staehelin et al. 1992).

EuNOD-CHT2 transcripts were detected at similar levels in the root and root nodule (Fig. 2) and strongly localized in the infected cells of the fixation zone (Fig. 4A and B). As mentioned above, the C-terminal amino acid sequence of EuNOD-CHT2 suggests that EuNOD-CHT2 protein is localized in the vacuole (Fig. 1). The functional significance of vacuolar localization of basic chitinases is thought to be the rapid release of large quantities of chitinases against pathogen infection. These results lead us to the hypothesis that EuNOD-CHT2 is induced because of the defense response against internal symbionts, external pathogens, or both.

Differential regulation of individual chitinase genes has been reported from many plant species (Collinge et al. 1993). Several reports also showed that chitinase isozymes have different enzymatic functions. For instance, 10 tobacco chitinases purified from the leaves reacting hypersensitively to *Tobacco mosaic virus* showed very different substrate specificities against chitin, chitosan, and the bacterial cell wall (Brunner et al. 1998). Kragh and colleagues (1996) also showed that chitinases purified from carrot culture medium have different capabilities to rescue carrot somatic embryo mutants. These previous results suggest that plant chitinases play different biological roles. Our results also showed the differential gene expression patterns of EuNOD-CHT1 and EuNOD-CHT2, suggesting different biological roles in the root nodule and during plant development.

In legume-*Rhizobium* symbiosis, a substance inducing nodule organogenesis was identified as Nod factor LCO, which is produced from a *Rhizobium* sp. (Lerouge et al. 1990). The presence of the root hair-deforming factor such as Nod factor was also recognized in the actinorhizal plant-*Frankia* system (VanGhelue et al. 1997), but the factor is genetically and structurally divergent from that of the *Rhizobium* factor (Crmonie et al. 1998, 1999). Especially, the root hair-deforming activity of the *Frankia* factor was not affected by chitinase treatment (Crmonie et al. 1999). These previous data suggest that the initial nodulation of the actinorhizal plant might not be under feedback regulation through the degradation of Nod factor by chitinases, shown in the alfalfa-*Rhizobium* system, in which the role of chitinases as a feedback regulator of nodulation was suggested (Staehelin et al. 1994, 1995).

Fig. 5. Expression patterns of EuNOD-CHT1 and EuNOD-CHT2 among root segments. Total RNA was extracted from the root meristem zone (MZ1), elongation zone (EZ), and maturation zone including secondary root (MZ2). The reverse-transcription-polymerase chain reaction product was separated on a 1.5% agarose gel, and DNA fragments transferred on nylon membrane were probed with the ³²P-labeled full insert of EuNOD-CHT1 clone and EuNOD-CHT2.

There are several reports that LCOs play a role in processes other than nodulation. A carrot embryo mutant arrested at the globular stage during somatic embryogenesis was rescued by rhizobial LCOs and a purified acidic chitinase (de Jong et al. 1992, 1993). Schlaman and colleagues (1997) showed that chitin oligosaccharides cotargeted with a root stele factor, uridine, without fatty acyl moiety, could induce cortical cell division in roots of *Vicia sativa*. From these previous reports, we suggest a putative endogenous role of EuNOD-CHT1 in the meristem region of the root nodule and the roots of actinorhizal plants. The EuNOD-CHT1 could be localized in the apoplastic space and may play a role in producing chitin-containing, signal molecules for cell division, developmental regulation, or both from the precursor molecules in the plant cell wall. This hypothesis can be supported by the fact that a basic chitinase-like protein from an embryogenic cell line of *Pinus caribaea* uses an arabinogalactan as an endogenous substrate (Domon et al. 2000). There are several indirect lines of evidence that chitin or chitinlike molecules, which might be used as substrates of chitinases, are also present in plants and animals as well as arthropods and fungi (Benhamou and Asselin 1989; Semino et al. 1996). Recently, van Hengel and colleagues (2001) showed direct evidence that chitinases can generate *N*-acetylglucosamine, glucosamine, or both, which are from arabinogalactan proteins (AGPs) and play crucial roles for the completion of carrot somatic embryogenesis. The chitinase-modified AGPs are not only capable of promoting embryogenesis from protoplasts but also are capable of reinitiating cell division in a subpopulation of nondividing protoplasts. The involvement of chitinases during cell wall loosening or modification has also been discussed in the flower development and seed germination of members of the family Solanaceae (Harikrishna et al. 1996; Takakura et al. 2000; Wu et al. 2001).

EuNOD-CHT1 transcripts were not detected in the roots by Northern hybridization (Fig. 2) but were detected at the root tip, including the meristem, and the maturation zone, including newly emerging secondary roots (Fig. 5). These results suggest that EuNOD-CHT1 transcripts may also be detected in the root apical meristem and play the same functional role as that in the root nodule, although the expression site should be confirmed by in situ hybridization. Although a relatively high level of EuNOD-CHT1 transcript was detected in healthy leaves (Fig. 2), we believe that defense is not the major function of EuNOD-CHT1 in the leaf. If it were, substantial amounts of the transcripts should have been detected in the root. In tobacco, a chitinase gene expression was induced during the development of roots and leaves (Neale et al. 1990). Gene expressions or enzyme activities of chitinase were also detected in several tissues of flower (Harikrishna et al. 1996; Leung 1992; Neale et al. 1990; Takakura et al. 2000), although the endogenous roles have not yet been demonstrated.

In conclusion, based on amino acid sequence features and differential gene expression of two chitinase clones, we suggest that the two genes may play different biological roles in the root nodule and during plant development. EuNOD-CHT2 may be involved in a defense response against internal symbionts, external pathogens, or both, while EuNOD-CHT1 may be involved in normal plant development as well as in a defensive role against external pathogens.

MATERIALS AND METHODS

Bacterial strain and plant material.

Frankia strain EuK1, which was isolated from a root nodule of *E. umbellata* and maintained in pure culture in defined propionate medium without nitrogen source, was used to nodulate *E. umbellata* seedlings (Kim et al. 1993). RNA for the con-

struction of the nodule cDNA library was isolated at various developmental stages from a 6-month-old plant after inoculation with *Frankia* strain EuK1. The uninoculated seedlings were used to isolate total RNA of leaves and roots. The harvested tissues were frozen in liquid nitrogen and stored at -80°C until needed.

Isolation of nucleic acids.

Total RNA and genomic DNA were isolated as described by Doyle and Doyle (1990), with some modification: polyvinylpolypyrrolidone was added during grinding with liquid nitrogen to remove phenolic compounds. RNase-free DNase (Promega, Madison, WI, U.S.A.) was used to remove genomic DNA contamination in an RNA sample during RNA purification.

Screening of a root nodule cDNA library.

The construction and screening of a nodule cDNA library was described previously by Kim and colleagues (1999). Nodule-specific or enhanced cDNA clones were isolated from 1×10^5 phage clones by the hybridization-competition method of Mangiarotti and colleagues (1981).

Cloning and sequence analysis.

Positive phage clones from primary and secondary differential screening were changed into phagemid clones according to the in vivo excision protocol of the manufacturer (Stratagene, La Jolla, CA, U.S.A.). Phagemid DNA was deleted unidirectionally with exonuclease III and S1 nuclease by using the double-stranded Nested Deletion Kit (Pharmacia Biotech, Uppsala, Sweden) based on the protocol of Henikoff (1984). The nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger et al. 1977) using *Taq* polymerase (Promega). The sequences were analyzed using PC-GENE (release 6.01; IntelliGenetics, Mountain View, CA, U.S.A.), DNASIS/PROSIS (version 6.01; Hitachi Software Engineering, Tokyo, Japan), and BLAST search program (Altschul et al. 1990).

RNA blot analysis.

Total RNA (20 μg) was separated on a 1% glyoxal gel and transferred to a Hybond-N membrane (Amersham, Buckinghamshire, U.K.) by the capillary blotting method (Sambrook et al. 1989). The blots were hybridized overnight with a ^{32}P -labeled DNA probe under the following condition: $6\times$ SSC (0.9 M NaCl plus 0.09 M sodium citrate, pH 7.0), $5\times$ Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) at 65°C . The hybridized blots were washed at 65°C with the salt concentration gradually decreasing to $0.5\times$ SSC (0.075 M NaCl plus 0.0075 M sodium citrate), 0.1% SDS and exposed to X-ray film.

RT-PCR.

RT-PCR was done to analyze the expression pattern of chitinase genes in root segments. The PCR primers were designed to specifically amplify the 3' untranslated region of two chitinase genes. The primer sequences are as follows: for EuNOD-CHT1, CHT1F = 5'-GCAACAACAGAGGCCTTT-3', CHT1R = 5'-TGGACTACTAAGCCCTC-3'; and for EuNOD-CHT2, CHT2F = 5'-TGGTGGGCTTGAATGTGGCA-3', CHT2R = 5'-CCTTCCACAATCCACACATAC-3'. Total RNA (1 μg) was used as a template for reverse transcription after RNase-free DNase (Promega) treatment. PCR cycling conditions were 95°C for 5 min for initial denaturation followed by 95°C for 1 min, 55°C (67°C for *nifH*) for 30 s, and 72°C for 45 s (for a total of 30 cycles), with 10 min of final extension at 72°C . Amplified PCR products were separated on 1.2% agarose gel, transferred to nylon membrane, and probed with inserts of pEuNOD-CHT1, pEuNOD-CHT2, and a ubiquitin clone (Kim and An 1999).

In situ hybridization.

Tissue preparation was performed essentially as described by Cox and Goldberg (1988). Root nodules were fixed overnight in FAA (50% [vol/vol] EtOH, 5% [vol/vol] glacial acetic acid, 10% [vol/vol] formaldehyde) under constant vacuum, dehydrated through a graded ethanol series, and embedded in Paraplast (Oxford, St. Louis, MO, U.S.A.). Tissue sections (10 μm thick) were applied to precleaned slide glasses treated with Vectabond (Vector Laboratories, Burlingame, CA, U.S.A.). Pretreatment, hybridization, and washing of slides were performed essentially as described by McKhann and Hirsch (1993). Antisense and sense RNA probes for in situ hybridization were prepared from linearized plasmids with DIG-11-rUTP (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Hybridization was performed at 42°C for at least 16 h. After posthybridization treatment and incubation with antidigoxigenin conjugated with alkaline phosphatase, substrate was added and color development was allowed in a dark cabinet for approximately 1 to 4 days. The sections were dehydrated through a graded ethanol series and then mounted with Permount (Fisher Scientific, Fair Lawn, NJ, U.S.A.).

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LITERATURE CITED

- Altschul, S. F., Gish, W., Miller, W., Meyers, E. W., and Lipman, D. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Baron, C., and Zambryski, P. C. 1995. The plant response in pathogenesis, symbiosis, and wounding: Variations on a common theme? *Annu. Rev. Genet.* 29:107-129.
- Bause, E. 1983. Structural requirements of *N*-glycosylation of proteins—Studies with proline peptides as conformational probes. *Biochem. J.* 209:331-336.
- Benhamou, N., and Asselin, A. 1989. Attempted localization of a substrate for chitinases in plant cells reveals abundant *N*-acetyl-*D*-glucosamine residues in secondary walls. *Biol. Cell* 67:341-350.
- Berry, A. M., and Sunell, L. A. 1990. The infection process and nodule development. Pages 61-81 in: *The Biology of Frankia and Actinorhizal Plants*. C. R. Schwintzer and J. D. Tjepkema, eds. Academic Press, New York.
- Brunner, F., Stintzi, A., Fritig, B., and Legrand, M. 1998. Substrate specificities of tobacco chitinases. *Plant J.* 14:225-234.
- Crmonie, H., Cournoyer, B., Maillet, F., Normand, P., and Fernandez, M. P. 1998. Genetic complementation of rhizobial *nod* mutants with *Frankia* DNA: Artifact or reality? *Mol. Gen. Genet.* 260:115-119.
- Crmonie, H., Debelle, F., and Fernandez, M. P. 1999. Structural and functional comparison of *Frankia* root hair deforming factor and rhizobia Nod factor. *Can. J. Bot.* 77:1293-1301.
- Collinge, D. B., Kragh, K. M., Mikkelsen, J. D., Nielsen, K. K., Rasmussen, U., and Vad, K. 1993. Plant chitinases. *Plant J.* 3:31-40.
- Cox, K. H., and Goldberg, R. B. 1988. Analysis of plant gene expression. Pages 1-35 in: *Plant Molecular Biology: A Practical Approach*. C. H. Shaw, ed. IRL Press, Oxford.
- de Jong, A. J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., van Kammen, A., and de Vries, S. C. 1992. A carrot somatic embryo mutant is rescued by chitinase. *Plant Cell* 4:425-433.
- de Jong, A. J., Heidstra, R., Spaik, H. P., Hartog, M. V., Meijer, E. A., Hendriks, T., Lo Schiavo, F., Terzi, M., Bisseling, T., van Kammen, A., and de Vries, S. C. 1993. *Rhizobium* lipooligosaccharides rescue a carrot somatic embryo mutant. *Plant Cell* 5:615-620.
- Domon, J. M., Neutelings, G., Roger, D., David, A., and David, H. 2000. A basic chitinase-like protein secreted by embryogenic tissues of *Pinus caribaea* acts on arabinogalactan proteins extracted from the same cell lines. *J. Plant Physiol.* 156:33-39.
- Doyle, J. J., and Doyle, J. I. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12:13-15.
- Goetting-Minesky, M. P., and Mullin, B. C. 1994. Differential gene-ex-

- pression in an actinorhizal symbiosis—Evidence for a nodule-specific cysteine proteinase. *Proc. Natl. Acad. Sci. U.S.A.* 91:9891-9895.
- Graham, L. S., and Sticklen, M. B. 1994. Plant chitinases. *Can. J. Bot.* 72:1057-1083.
- Hanfrey, C., Fife, M., and Buchanan-Wollaston, V. 1996. Leaf senescence in *Brassica napus*: Expression of genes encoding pathogenesis-related proteins. *Plant Mol. Biol.* 30:597-609.
- Harikrishna, K., Jampates-Beale, R., Milligan, S. B., and Gasser, C. S. 1996. An endochitinase gene expressed at high levels in the stylar transmitting tissue of tomatoes. *Plant Mol. Biol.* 30:899-911.
- Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoint for DNA sequencing. *Gene* 28:351-359.
- Hirsch, A. M. 1992. Developmental biology of legume nodulation. *New Phytol.* 122:211-237.
- Kim, H. B. 1998. Structures and expression patterns of cDNA clones encoding asparagine synthetase, chitinase, and polyubiquitin from the root nodule of *Elaeagnus umbellata*. Ph.D. thesis. Seoul National University, Seoul, Korea.
- Kim, H. B., and An, C. S. 1999. Isolation and characterization of a cDNA clone encoding polyubiquitin from the root nodule of *Elaeagnus umbellata*. *Can. J. Bot.* 77:1270-1278.
- Kim, H. B., Lee, S. H., and An, C. S. 1999. Isolation and characterization of a cDNA clone encoding asparagine synthetase from root nodules of *Elaeagnus umbellata*. *Plant Sci.* 149:85-94.
- Kim, S. C., Ku, C. D., Park, M. C., Kim, C. H., Song, S. D., and An, C. S. 1993. Isolation of symbiotic *Frankia* EulK1 strain from root nodule of *Elaeagnus umbellata*. *Korean J. Bot.* 36:177-182.
- Kragh, K. M., Hendricks, T., De Jong, A. J., Lo Schiavo, F., Bucherna, N., Hojrup, P., Mikkelsen, J. D., and de Vries, S. C. 1996. Characterization of chitinases able to rescue somatic embryos of the temperature-sensitive carrot variant *ts11*. *Plant Mol. Biol.* 31:631-645.
- Laplace, L., Ribeiro, A., Franche, C., Duhoux, E., Auguy, F., Bogusz, D., and Pawlowski, K. 2000. Characterization of a *Casuarina glauca* nodule-specific subtilisin-like protease gene, a homolog of *Alnus glutinosa* ag12. *Mol. Plant-Microbe Interact.* 13:113-117.
- Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C., and Denarie, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulfated and acylated glucosamine oligosaccharide signal. *Nature* 344:781-784.
- Leung, D. W. M. 1992. Involvement of plant chitinase in sexual reproduction of higher-plants. *Phytochemistry* 31:1899-1900.
- Mangiarotti, G., Chung, S., Zuker, C., and Lodish, H. F. 1981. Selection and analysis of cloned developmentally-regulated *Dictyostelium discoideum* genes by hybridization-competition. *Nucleic Acids Res.* 9:947-963.
- McKhann, H. I., and Hirsch, A. M. 1993. *In situ* localization of specific mRNAs in plant tissues. Pages 179-205 in: *Methods in Plant Molecular Biology and Biotechnology*. B. R. Glick and J. E. Thompson, eds. CRC Press, Boca Raton, FL, U.S.A.
- Neale, A. D., Wahleithner, J. A., Lund, M., Bonnett, H. T., Kelly, A., Meekswagner, D. R., Peacock, W. J., and Dennis, E. S. 1990. Chitinase, beta-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. *Plant Cell* 2:673-684.
- Neuhaus, J. M., Sticher, L., Meins, F., and Boller, T. 1991. A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. *Proc. Natl. Acad. Sci. U.S.A.* 88:10362-10366.
- Neuhaus, J. M., Fritig, B., Linthorst, H. J. M., Meins, F., Mikkelsen, J. D., and Ryals, J. 1996. A revised nomenclature for chitinase genes. *Plant Mol. Biol. Rep.* 14:102-104.
- Pawlowski, K., Twigg, P., Dobritsa, S., Guan, C., and Mullin, B. C. 1997. A nodule-specific gene family from *Alnus glutinosa* encodes glycine- and histidine-rich proteins expressed in the early stages of actinorhizal nodule development. *Mol. Plant-Microbe Interact.* 10:656-664.
- Payne, G., Ahl, P., Moyer, M., Harper, A., Beck, J., Meins, F., and Ryals, J. 1990. Isolation of complementary DNA clones encoding pathogenesis-related protein-P and protein-Q, 2 acidic chitinases from tobacco. *Proc. Natl. Acad. Sci. U.S.A.* 87:98-102.
- Ribeiro, A., Akkermans, A. D. L., van Kammen, A., Bisseling, T., and Pawlowski, K. 1995. A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. *Plant Cell* 7:785-794.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467.
- Schlaman, H. R. M., Gisela, A. A., Quaedyvlieg, N. E. M., Bloembergen, G. V., Lugtenberg, B. J. J., Kijne, J. W., Potrykus, I., Spaik, H. P., and Sautter, C. 1997. Chitin oligosaccharides can induce cortical cell division in roots of *Vicia sativa* when delivered by ballistic microtargeting. *Development* 124:4887-4895.
- Schubert, K. R. 1986. Products of biological nitrogen-fixation in higher-plants—Synthesis, transport, and metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 37:539-574.
- Semino, C. E., Specht, C. A., Raimondi, A., and Robbins, P. W. 1996. Homologs of the *Xenopus* developmental gene DG42 are present in zebrafish and mouse and are involved in the synthesis of Nod-like chitin oligosaccharides during early embryogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 93:4548-4553.
- Shinshi, H., Neuhaus, J. M., Ryals, J., and Meins, F. 1990. Structure of a tobacco endochitinase gene—Evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. *Plant Mol. Biol.* 14:357-368.
- Staehelin, C., Muller, J., Mellor, R. B., Wiemken, A., and Boller, T. 1992. Chitinase and peroxidase in effective (*fix*⁺) and ineffective (*fix*⁻) soybean nodules. *Planta* 187:295-300.
- Staehelin, C., Schuitze, M., Kondorosi, E., Mellor, R. B., Boller, T., and Kondorosi, A. 1994. Structural modifications in *Rhizobium meliloti* Nod factors influence their stability against hydrolysis by root chitinases. *Plant J.* 5:319-330.
- Staehelin, C., Schultze, M., Kondorosi, E., and Kondorosi, A. 1995. Lipo-chitoooligosaccharide nodulation signals from *Rhizobium meliloti* induce their rapid degradation by the host-plant alfalfa. *Plant Physiol.* 108:1607-1614.
- Takakura, Y., Ito, T., Saito, H., Inoue, T., Komari, T., and Kuwata, S. 2000. Flower-predominant expression of a gene encoding a novel class I chitinase in rice (*Oryza sativa* L.). *Plant Mol. Biol.* 42:883-897.
- Vance, C. P. 1983. *Rhizobium* infection and nodulation: A beneficial plant disease? *Annu. Rev. Microbiol.* 37:399-424.
- VanGhelue, M., Lovaas, E., Ringo, E., and Solheim, B. 1997. Early interactions between *Alnus glutinosa* and *Frankia* strain Ar13. Production and specificity of root hair deformation factor(s). *Physiol. Plant.* 99:579-587.
- van Hengel, A. J., Tadesse, Z., Immerzeel, P., Schols, H., van Kammen, A., and de Vries, S. C. 2001. N-acetyl glucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. *Plant Physiol.* 125:1880-1890.
- Vasse, J., Debilly, F., and Truchet, G. 1993. Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. *Plant J.* 4:555-566.
- Verburg, J. G., Smith, C. E., Lisek, C. A., and Huynh, Q. K. 1992. Identification of an essential tyrosine residue in the catalytic site of a chitinase isolated from *Zea mays* that is selectively modified during inactivation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. *J. Biol. Chem.* 267:3886-3893.
- Wu, C. T., Leubner-Metger, G., Meins, F., and Bradford, K. 2001. Class I β -1,3-glucanase and chitinase are expressed in the micropylar endosperm of tomato seeds prior to radicle emergence. *Plant Physiol.* 126:1299-1313.

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