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Constitutive Expression of Two Endochitinases from Root Nodules of *Elaeagnus umbellata* Confers Resistance on Transgenic *Arabidopsis* Plants against the Fungal Pathogen *Botrytis cinerea*

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Plant chitinases have been known as pathogenesis-related (PR) proteins, but recent studies suggest that they play functional roles during normal plant growth and development. We previously isolated two cDNA clones encoding endochitinases, *EuNOD-CHT1* and *-CHT2*, from the root nodules of *Elaeagnus umbellata*. These genes show differential expression patterns, with the *EuNOD-CHT1* gene being active in the root nodules and meristems, while *EuNOD-CHT2* is preferentially expressed in the infected cells of those nodules. To elucidate the functional roles of these two endochitinases, we have now constitutively expressed each gene in a heterologous plant system, *Arabidopsis thaliana*. Stable inheritance and expression of the transgenes were confirmed by genomic Southern hybridization and RT-PCR. Our transgenic plants did not differ morphologically from the wild types. However, constitutive expression of *EuNOD-CHT1* and *-CHT2* in *Arabidopsis* resulted in increased resistance against a fungal pathogen, *Botrytis cinerea*, but not against a bacterial agent, *Pseudomonas syringae* pv. Tomato DC3000. Expression levels were enhanced by both wounding and jasmonic acid treatments (for *EuNOD-CHT1*), or by jasmonic acid only (for *EuNOD-CHT2*). These data suggest that *EuNOD-CHT1* and *-CHT2* primarily play defensive roles during root nodule development in *E. umbellata*.

Keywords: Arabidopsis thaliana, Botrytis cinerea, chitinase, constitutive expression, pathogen resistance, root nodule

Chitinases (EC 3.2.1.14) are classified as glycosyl hydrolases that catalyze the degradation of chitin, an insoluble linear β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNac) residues. Various chitinases inhibit the growth of many fungi *in vitro* by causing lysis of the hyphal tips, especially in combination with β-1,3-glucanase. For example, a chitinase induced by fungal infection has been shown to accumulate *in planta* around the fungal hyphae walls at infection sites (see reviews by Collinge et al., 1993; Passarinho and de Vries, 2002). Enhanced chitinase levels can significantly reduce the susceptibility of transgenic plants to certain pathogens, providing an excellent tool for improving pest control (Broglie et al., 1991; Jach et al., 1995).

Chitinase genes have been identified in bacteria, fungi, yeast, animals, and plants (see review by Passarinho and de Vries, 2002). Plant chitinases have been well characterized as pathogenesis-related (PR) proteins because their activity is induced not only by pathogen infections but also by various sources of stress, e.g., wounding, phytohormones, heavy metals, and elicitors (see reviews by Collinge et al., 1993; Graham and Sticklen, 1994). Through many analogies between pathogenesis and symbiosis, symbiotic bacteria are also now considered to be similar to parasites (see reviews by Vance, 1983; Baron and Zambryski, 1995).

Defense-related genes, including various plant chitinases, are induced during the nodulation process that occurs in legume-*Rhizobium* and actinorhizal plant-*Frankia* symbioses (see review by Baron and Zambryski, 1995; Kim and An, 2002; Kim et al., 2003). However, these chitinases might also play functional roles during normal growth and development, as seen in the rescue of a carrot somatic embryo mutant by a purified acidic chitinase (de Jong et al., 1992; Kragh et al., 1996), the increased expression levels of chitinases during normal plant development (Harikrishna et al., 1996; van Hengel et al., 1998; Takakura et al., 2000; Passarinho et al., 2001; Wu et al., 2001), and the appearance of chitinases during leaf senescence (Hanfrey et al., 1996). Moreover, Zhong et al. (2002) have isolated an *Arabidopsis* chitinase mutant that shows many developmental defects, such as the ectopic deposition of lignin, aberrant cell shapes, and

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overproduction of ethylene. However, the expression level of the chitinase gene that causes these developmental abnormalities is not altered by wounding, salicylic acid, elicitors, and ethylene, further demonstrating that its expression is also essential for normal growth and development. In other transgenics, up- or down-regulation of chitinase genes has no effect on resistance or susceptibility to fungal infection (Neuhaus et al., 1991; Samac and Shah, 1994). Therefore, it is still unclear whether the primary functional role of certain chitinases is defense against pathogen attack or something else.

We previously identified two cDNA clones, *EuNODCHT1* and *-CHT2*, that encode endochitinases (Kim and An, 2002). These were isolated from a root nodule cDNA library of *Elaeagnus umbellata,* an actinorhizal plant that interacts with *Frankia* to form root nodules. *EuNOD-CHT1* transcripts are strongly detected in the roots and in the root-nodule meristems. In contrast, *EuNODCHT2* transcripts are found in root-nodule cells infected with *Frankia*. Based on *in situ* localization and other supporting data, we suggest that the first gene may play a non-defensive role, whereas the second has a protective function. In this continuing study, we generated transgenic plants constitutively expressing those endochitinases, and analyzed their traits to elucidate their functional roles during normal plant development and in defense responsiveness.

MATERIALS AND METHODS

Plant Material and Growth Conditions

A. thaliana (L.) Heynh. ecotype Columbia (Col-0) was used as a wild type for transformation. For *in vivo* culture, seeds were surface-sterilized (20% Clorox and 0.1% Triton X-100) for 10 min, then washed five times with sterile distilled water. They were sprinkled on 0.8% agar-solidified media containing 1X Murashige and Skoog (1962) salts and 1% sucrose (pH 5.8 with KOH). For the selection of transgenic plants, MS media were supplemented with 40 μg mL^{-1} kanamycin or 80 μg mL^{-1} gentamycin. Seeds were planted on pre-moistened soil (BioPlug #2; Hungnong Seeds, Korea) mixed with vermiculite. The pots were covered with plastic wrap and cold-treated at 4°C for 2 d before being transferred to a growth chamber (16-h photoperiod and 24°C/18°C day/night temperature). The plastic wrap was removed after the first leaves appeared. Pots were sub-irrigated in distilled water as required.

Isolation of Nucleic Acids

Total RNA was purified using TRI Reagent (Sigma, USA). Total genomic DNAs from *A. thaliana* and *E. umbellata* were purified as described by Doyle and Doyle (1990), with the following modification: PVPP was added during grinding with liquid nitrogen to remove phenolic compounds.

Overexpression of *EuNOD-CHT1* and *-CHT2* in *Arabidopsis*

The full-length cDNA clones for two chitinases, *EuNOD-CHT1* and *-CHT2* (Kim and An, 2002), were digested with *Bam*HI and *Xba*I. Each DNA fragment was sub-cloned between the CaMV 35S promoter and the poly(A) signal of the pRT101 vector (Töpfer et al., 1987). *Pst*I fragments containing the overexpression cassettes from pRT101 were then sub-cloned into binary vectors pPZP221 (for *EuNOD-CHT1*) and pPZP111 (for *EuNOD-CHT2*) (Hajdukiewicz et al., 1994). The binary vectors containing these overexpression cassettes were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation, and introduced into *A. thaliana* ecotype Columbia via the floral-dip method (Clough and Bent, 1998). Transgenic plants (T_1 generation) were selected on MS plates containing either gentamycin (80 μg mL^{-1}) or kanamycin (40 μg mL^{-1}). Homozygous transgenic lines that overexpressed *EuNOD-CHT1* and *-CHT2* were selected from the T_3 generation.

DNA and RNA Gel Blot Analyses

For the DNA analysis, genomic DNA (3 μg) purified from 12-d-old *Arabidopsis* seedlings was digested with *Hind*III, separated on a 1% agarose gel, and transferred to a nylon membrane (Amersham, UK) by the capillary blotting method (Sambrook et al., 1989). For the RNA analysis, total RNA (20 μg) was separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. The DNA or RNA blots were hybridized overnight with a ^{32}P-labeled DNA probe under the following conditions: 6X SSC [0.9 M NaCl and 0.09 M sodium citrate (pH 7.0)] 5X Denhardt's solution, and 0.1% SDS, at 63°C. The hybridized blots were washed at 63°C, with the salt concentration gradually decreasing to 1X SSC, and then exposed to X-ray film (Fuji, Japan).

Reverse Transcription-Polymerase Chain Reaction

Purified total RNA (2 μg) was used for first-strand cDNA synthesis with Superscript II RNase H reverse transcriptase (Gibco BRL, USA). Primers and RT-PCR conditions were described previously (Kim and An, 2002). Briefly, the amplification was conducted as follows: 5 min for initial denaturation at 95°C; followed by 95°C for 1 min, 55°C for 30 s, and 72°C for 45 s (total of 27 cycles); with 10 min of final extension at 72°C. The amplified PCR products were separated on a 1.2% agarose gel.

Infection with Bacterial and Fungal Phytopathogens

To infect plants with *Pseudomonas syringae* pv. Tomato DC3000, we first cultured the bacterial strain for 2 d at 28°C on a solid KB medium (10 g Difco proteous peptone #3, 0.75 g K_2HPO_4, 0.75 g $MgSO_4 \cdot 7H_2O$, 7.5 mL glycerol, and 7.5 g agar per 500 mL). A single colony was then inoculated to a liquid KB medium. Bacterial cultures were harvested by centrifugation, suspended in 10 mM $MgCl_2$, and diluted to 10^8 cfu mL^{-1}. The diluted bacterial solution (5 μL) was infiltrated into the abaxial sides of the 5th and 6th leaves using a syringe without needle.

To infect the plants with a fungal organism, we cultured *Botrytis cinerea*, a pathogen for cruciferous species, in a PDA medium (19.5 g potato dextrose agar per 500 mL) under a 12-h photoperiod for 7 d at 20°C. Spores were harvested and diluted into a 10^5 cfu mL^{-1} solution of 20% V8 juice and 0.1 M K_2HPO_4. Plants were sprayed with this diluted spore solution, then cultured in a humidified growth chamber for 7 d at 20°C, and further cultured in a growth chamber for 1 d at 18°C. Disease symptoms were inspected visually.

Isolation of Promoter Regions for *EuNOD-CHT1* and *-CHT2*

The promoter regions for *EuNOD-CHT1* and *-CHT2* were cloned according to manufacturer's instructions for the BD GenomeWalker Universal Kit (Clontech, USA). Gene-specific primers were designed from the cDNA clones as follows: for *EuNOD-CHT1*, CHT1-GSP1, 5'-CAGTAACCCCATTGACTGCAACACAAC-3' and CHT1-GSP2, 5'-CTGAACACAGAGCACCCTCT AATTGTT-3'; and for *EuNOD-CHT2*, CHT2-GSP1, 5'-GCAGTATTCATCAGTAGTGCCACACCA-3' and CHT2-GSP2, 5'- AGTGCCACACCAACCATATTGGC-TAC-3'. The amplified PCR products were cloned into the pGEM T-easy vector (Promega, USA), and nucleotide sequences from the cloned DNA fragments were determined using a BigDye Terminator Cycle Ready Reaction Kit (Applied Biosystems, USA) and an ABI 3730 DNA Analyzer (Hitachi, Japan). The *cis*-acting regulatory DNA elements of the promoter regions were predicted via PlantCARE (http://oberon.fvms.ugent.be: 8080/PlantCARE).

Phytohormone and Wounding Treatments

Leaves were detached from 3-month-old *E. umbellata* plants and incubated under continuous light for 12 h in a liquid MS medium [1X MS basal salts, 1% sucrose, 0.5 g L^{-1} MES, and 0.01% Tween 20 (pH 5.8)] that contained 10 μM of methyl jasmonate (JA), salicylic acid (SA), or ACC (1-aminocyclopropane-1-carboxylic acid). To wound the tissues, detached leaves were dissected with a sterile razor blade and incubated in a liquid MS medium for 12 h. As the control, detached leaves were incubated in a liquid MS medium for 12 h.

RESULTS

Generation of Transgenic Plants Constitutively Expressing *EuNOD-CHT1* and *-CHT2*

To elucidate the functional roles of two endochitinase genes from the root nodules of *E. umbellata*, we constitutively expressed *EuNOD-CHT1* and *-CHT2* in wild-type *Arabidopsis* (Fig. 1A). Through segregation analysis for marker genes, we selected four and seven independent transgenic lines containing single-copy transgenes from the T_2 generation of *EuNOD-CHT1* and *-CHT2*, respectively. To check for their stable expression in the transgenic lines, total RNA was isolated from gentamycin- or kanamycin-resistant seedlings of the segregating T_2 generation and subjected to RT-PCR analysis (Fig. 1B). Several lines were identified that constitutively expressed each gene. Based on these RT-PCR results, we chose two independent transgenic lines from each construct for further analysis: Lines #4 and #11 for *EuNOD-CHT1*, and Lines #7 and #9 for *EuNOD-CHT2*. To again confirm their copy numbers, total genomic DNA was isolated from the homozygous lines of the T_3 generation and subjected to DNA gel blot analysis. Genomic Southern hybridization revealed that each transgenic line was independent and contained a single copy of the transgene on the chromosome (Fig. 1C).

Transgenic lines that constitutively expressed *EuNOD-CHT2* either at the seedling or the mature stage showed no morphological changes when compared with the wild-type plants. However, expression of *EuNOD-CHT1* in *Arabidopsis* resulted in an increase in the number of lateral roots as well as greater vegetative growth compared with either the wild-type controls or the *EuNOD-CHT2* transgenic lines (Table 1).

Resistance of Transgenic Plants to Phytopathogen Infections

Constitutive expression of PR (pathogenesis-related) genes increases plant resistance against infecting phytopathogens (Melchers and Stuiver, 2000). Chitinase, as a hydrolytic enzyme and member of the extensively studied family of PR proteins, is capable of

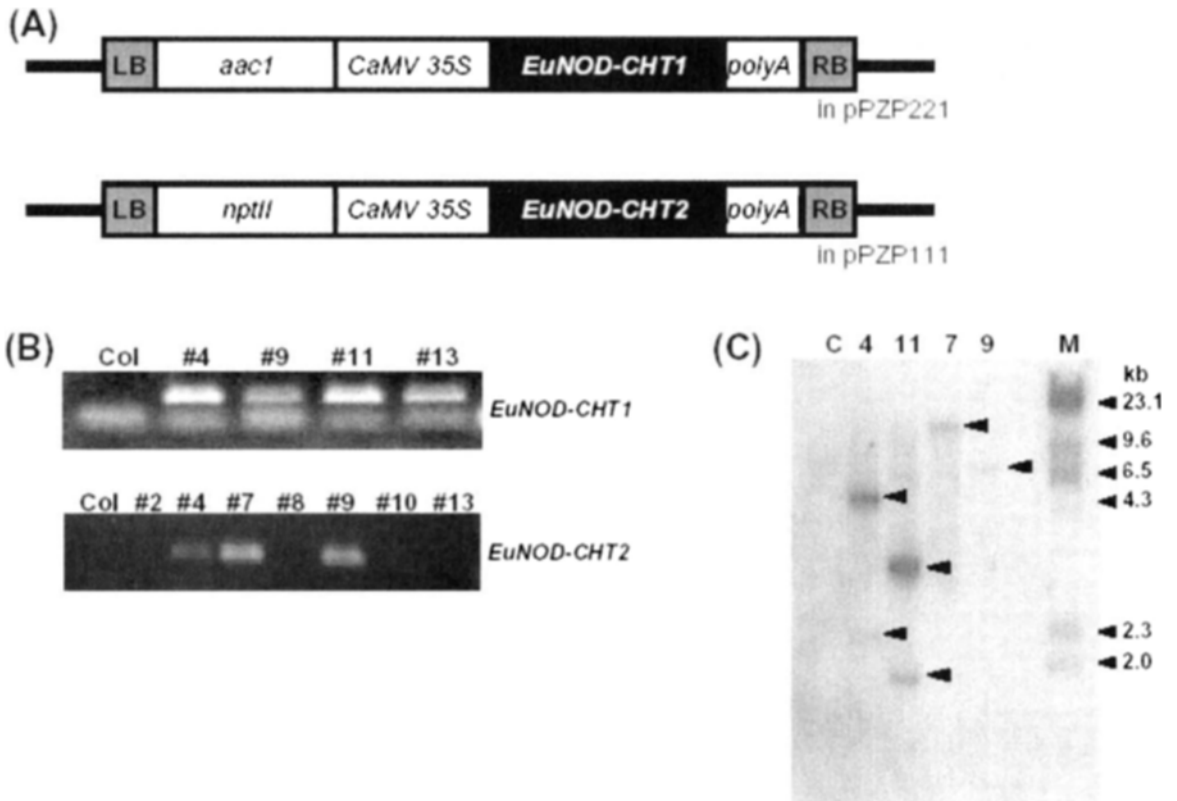

Figure 1. Generation of transgenic plants overexpressing *EuNOD-CHT1* and *-CHT2* in wild-type *Arabidopsis*. **A**, Constructs to overexpress *EuNOD-CHT1* and *-CHT2* in *Arabidopsis*. Selectable marker genes *aacC1* and *nptII* confer resistance for gentamycin and kanamycin, respectively. Constructs were used to transform *A. tumefaciens* GV3101 strain. *Arabidopsis* plants were transformed by floral-dip method. **B**, RT-PCR analysis of transgenic plants. 'Col' and numbers indicate wild-type plant and different transgenic lines, respectively. **C**, Genomic Southern hybridization for transgenic plants. Arrowheads indicate hybridization signals. *EuNOD-CHT1* clone contained *Hin*dIII restriction site in coding region, resulting in two signals. M, λ/*Hin*dIII marker; C, wild type, Colombia; 4 and 11, lines #4 and #11 for *35S:EuNOD-CHT1*; 7 and 9, lines #7 and #9 for *35S:EuNOD-CHT2*.

Table 1. Comparison of number of lateral roots, and fresh and dry weights in selected transgenic lines constitutively expressing endochitinases in *Arabidopsis*.

	Wild type	35S:EuNOD-CHT1		35S:EuNOD-CHT2	
		#4	#11	#7	#9
No. of lateral roots[a]	6.07 ± 0.88	9.09 ± 1.72	12.20 ± 2.18	7.00 ± 1.48	5.62 ± 1.21
Fresh weight[b] (mg)	246.7 ± 22.7	393.3 ± 24.5	380 ± 44.6	270 ± 5.4	283.3 ± 13.7
Dry weight[b] (mg)	21.02 ± 1.90	31.15 ± 1.57	44.42 ± 6.46	25.47 ± 4.26	23.47 ± 1.09

[a] Number of lateral roots 9 d after germination; [b] Fresh and dry weights 40 d after germination. Each value is mean (± standard error) of 20 measurements.

degrading the major cell-wall components of most filamentous fungi. To elucidate a defensive function for *EuNOD-CHT1* and/or *-CHT2* as PR genes, we tested the infection resistance of transgenic plants.

P. syringae pv. tomato DC3000 is a bacterial strain highly virulent on *A. thaliana* ecotype Col-0 (Whalen et al., 1991). When compared with the wild types, our transgenic plants overexpressing either *EuNOD-CHT1* or *-CHT2* exhibited no notable resistance to this strain (data not shown).

A gray mold, *B. cinerea,* which contains chitin in its cell walls, infects a broad range of species, including *Arabidopsis* (Koch and Slusarenko, 1990). Leaves of our wild-type plants were completely collapsed and finally died 8 d after inoculation with this fungal pathogen (Fig. 2A). In contrast, transgenic lines constitutively expressing either *EuNOD-CHT1* (Fig. 2B and C) or *-CHT2* (Fig. 2D and E) showed remarkable resistance. Our previous results suggested that EuNOD-CHT1 and -CHT2 could be localized into the apoplastic spaces and vacuoles, respectively (Kim and An, 2002). With regard to this *Botrytis* infection, we believe that EuNOD-CHT1, an apoplastic chitinase, may act directly by inhibiting growth of the invading fungal hyphae and/or indirectly by releasing fungal cell wall fragments, which act as elicitors of other defense-related genes, including additional chitinase genes. Likewise, EuNOD-CHT2, a vacuolar chitinase, may play a role in inhibiting fungal hyphae growth upon cell collapse, as has been reported with other plant chitinases (for reviews, see Collinge et al., 1993; Graham and Sticklen, 1994).

Wounding and JA Inducibilities of *EuNOD-CHT1* and *-CHT2* Expression

Chitinase genes can be regulated by various biotic and abiotic factors. Defense responses in plant-pathogen interactions are mediated by various phytohormones, such as ethylene, jasmonic acid, and salicylic acid (for review, see Melchers and Stuiver, 2000). To better understand the functional roles and regulation of expression for our two endochitinase genes, we isolated their corresponding promoter regions and predicted putative *cis*-acting DNA regulatory elements. Using the genome-walking method, we obtained 683 bp and 813 bp of the 5 promoter regions upstream of the start codon (ATG) for *EuNOD-CHT1* and *EuNOD-*

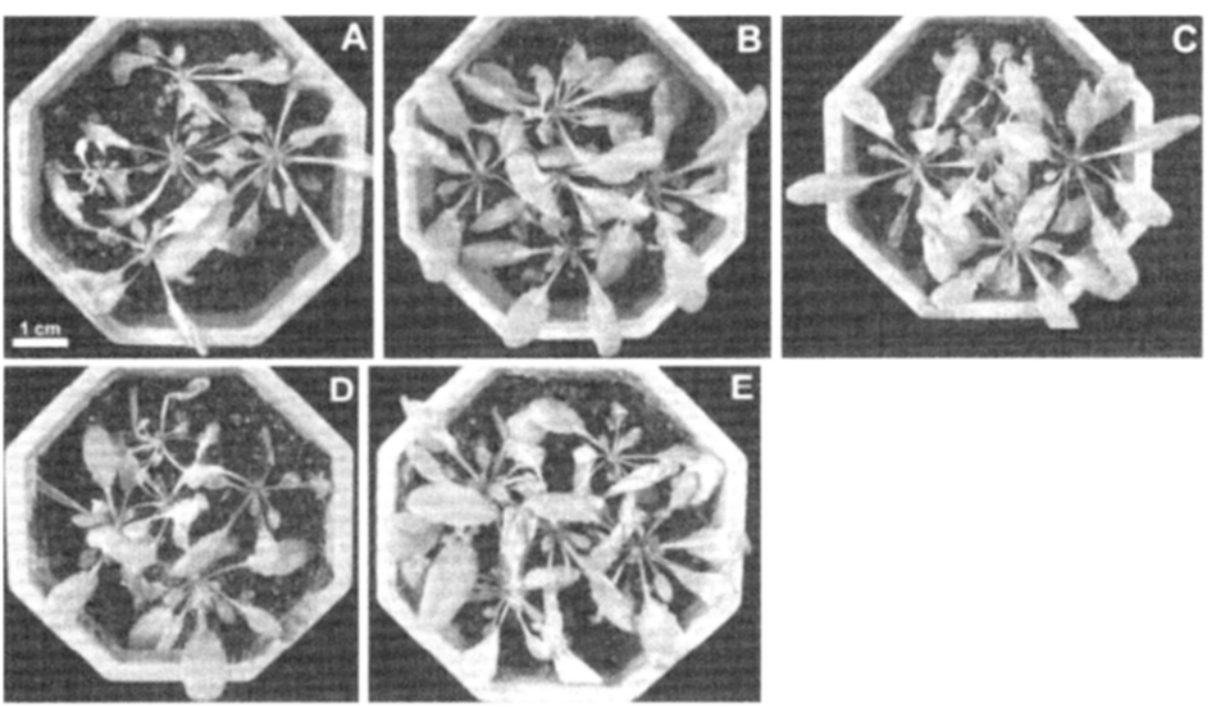

Figure 2. Resistance of transgenic plants to infection by fungal pathogen *B. cinerea*. Plants were photographed 8 d after inoculation. Pathogen resistance test was performed with 10 wild-type plants and 10 each for transgenic lines. All lines showed noticeable resistance compared with wild type. **A**, wild-type plants. **B** and **C**, transgenic plants (lines #4 and #11, respectively) constitutively expressing *EuNOD-CHT1*. **D** and **E**, transgenic plants (lines #7 and #9, respectively) constitutively expressing *EuNOD-CHT2*.

CHT2, respectively. In each promoter region, we identified several putative TATA and CAAT boxes, which are found in most eukaryotes and are basic *cis*-elements essential for transcription by RNA pol II (Fig. 3). Putative upstream *cis*-regulatory elements were also found in each promoter region -- a wound-responsive element in the *EuNOD-CHT1* promoter, and a JA-responsive element in the *EuNOD-CHT2* promoter (Fig. 3).

To further clarify that the upstream regulatory motifs found in those promoter regions are meaningful, we performed gel blot analysis using total RNA from leaves treated with wounding or stress-related phytohormones. The expression level for *EuNOD-CHT2* was increased only by JA (Fig. 4), whereas *EuNOD-CHT1* expression was induced by both wounding and JA (Fig. 4), even though the JA-responsive element was not found in its 5'-upstream region (Fig. 3A). We suppose that the promoter region obtained in this study was too short to have included that element. All these results suggest that expression of both endochitinase genes is differentially regulated by wounding and stress-related phytohormones. Nevertheless, transgenic studies using these promoter fragments fused to reporter genes will give us clearer evidence for whether the upstream regulatory elements found in those promoter regions are functional.

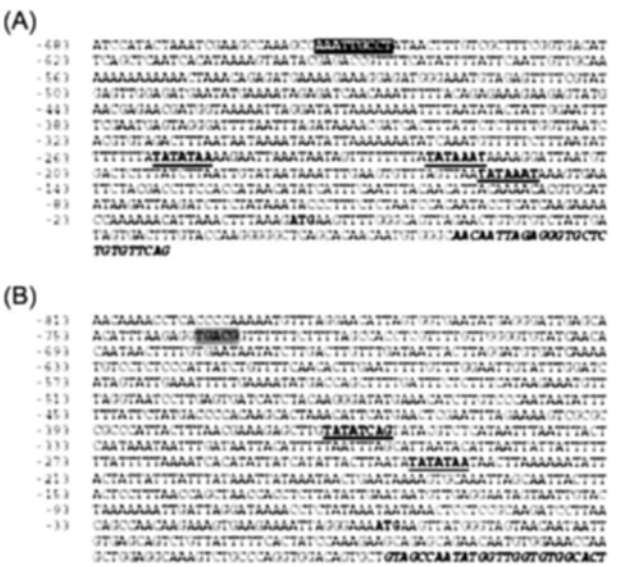

Figure 3. Partial promoter regions for *EuNOD-CHT1* **(A)** and *-CHT2* **(B)**. Reverse primers CHT1-GSP2 and CHT2-GSP2 used to amplify 5' upstream region are bolded and italicized, respectively. Putative TATA elements are bolded and underlined. Black box in **(A)** indicates putative wound-response element. Gray box in **(B)** indicates putative *cis*-acting regulatory element involved in JA-responsiveness. Bold ATGs in both **(A)** and **(B)** indicate translation start codons.

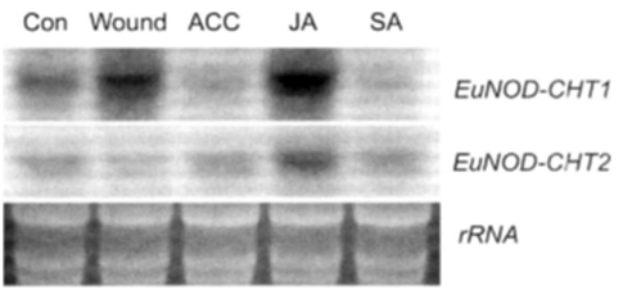

Figure 4. Effects of wounding and phytohormone treatments on expression levels of *EuNODCHT1* and *-CHT2* in leaves of *E. umbellata*. ACC, 1-aminocyclopropane-1- carboxylic acid; JA, methyl jasmonate; SA, salicylic acid.

DISCUSSION

In this paper, to elucidate the functional roles of two endochitinase genes, *EuNODCHT1* and *-CHT2*, which were previously isolated from the root nodules of *E. umbellata*, we generated *Arabidopsis* transgenic plants that manifest their constitutive expression. Those plants show noticeable resistance against the fungus *B. cinerea* (Fig. 2). Expression of both endochitinase genes was positively regulated by wounding and/or jasmonic acid (Fig. 4), the latter serving as a signaling molecule to turn on defense responses during plant-pathogen interactions. Putative wounding- and/or JA-responsive elements were also found in the upstream regulatory regions of the genes (Fig. 3). Based on these results, we suggest that both endochitinases act as PR proteins to inhibit hyphal growth in the infecting fungal pathogens. In terms of expression patterns in the root nodules of *E. umbellata*, *EuNOD-CHT1* might be involved in protecting the nodule meristems from external pathogens. In contrast, enhanced expression of *EuNOD-CHT2* in cells infected with symbiotic *Frankia* may be attributed to a defense response against infecting symbionts (Kim and An, 2002).

Interestingly, constitutive expression of *EuNOD-CHT1* in *Arabidopsis* resulted in increased lateral rooting and enhanced vegetative growth (Table 1). Plant chitinases have long been believed to play roles during normal growth and development. For instance, a loss-of-function mutation for an *Arabidopsis* chitinase causes severe reductions in growth as well as abnormalities in other developmental aspects (Zhong et al., 2002). Likewise, constitutive expression of an endochitinase gene from *Streptomyces albidoflavus* in tomato positively affects flowering time as well as the

number of flowers and fruit on transgenic plants (Gongora and Broadway, 2002). In fact, constitutive expression of *EuNOD-CHT1* in our *Arabidopsis* plants accelerated the relative emergence rate of primary inflorescence (data not shown), and was related to increased fresh and dry weights 40 d after germination (Table 1). Therefore, we cannot rule out the possibility that *EuNOD-CHT1* plays roles in normal plant growth and development. Further detailed experiments are needed to clarify this potential role.

One of the primary goals of crop breeding programs is to obtain crops with multiple traits. We have demonstrated here that the *EuNOD-CHT1* gene could be a valuable resource for promoting plant growth as well as increasing resistance to infections by fungal pathogens.

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