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Isolation and characterization of *cgchi3*, a nodule-specific gene from *Casuarina glauca* encoding a class III chitinase

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Chitinases (EC 3.2.1.14) catalyse the hydrolysis of chitin, a homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine residues. Plant chitinases are involved in a wide variety of processes; in particular, their expression has been found to be enhanced in symbiotic and pathogenic plant–microbe interactions. During this work we have cloned and characterized a gene encoding a class III chitinase from actinorhizal nodules of *Casuarina glauca* (*cgchi3*). CGCHI3 was found to be encoded by a single gene that was specifically activated in nodules as compared with uninoculated control roots and leaves. The expression of this gene was further enhanced in nodules after salicylic acid treatment and completely repressed after wounding. In situ hybridisation analysis revealed that *cgchi3* is an early nodulin gene, being expressed in the meristem and in the uninfected cortical cells of young nodules. Based on the obtained results we suggest that this gene is involved in nodule development. This is the first report on a class III chitinase coding gene that is specifically activated during actinorhizal symbiosis.

Introduction

Actinorhizal nodules are induced by actinomycetes of the genus *Frankia* on the roots of dicotyledonous plants belonging to eight different plant families, mostly trees or woody shrubs, collectively called actinorhizal plants. Inside the nodules, bacteria are hosted within cortical cells and provide the plant with fixed atmospheric nitrogen. In exchange, the plant provides the bacteria with carbon and energy sources that are essential to sustain the process of nitrogen fixation. Actinorhizal nodules are coralloid structures consisting of multiple lobes, which represent modified lateral roots, with a central vascular system, without root caps, and with infected cortical cells (Berry and Sunell 1990). Because of the presence of an apical meristem, actinorhizal nodule lobes have an indeterminate growth pattern and four different zones can be distinguished in the nodule cortex (Ribeiro et al. 1995): (1) meristematic zone (zone 1); (2) infection zone (zone 2), where some cortical cells originating from the meristem are infected by *Frankia*; (3) fixation zone (zone 3), with mature infected cells, containing differentiated *Frankia* vesicles, the place of

Abbreviations – AGPs, ArabinoGalactan proteins; AMV, avian myeloblastosis virus; B/P, *Casuarina glauca* genomic DNA digested with *Bam*HI/*Pst*I; C−, negative control; cDNA, complementary DNA; Cg, *Casuarina glauca* genomic DNA; DIG, digoxigenin; ENOD, early NODulin; FCT, Fundação para a Ciência e Tecnologia; JA, jasmonic acid; NFs, Nod factors; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; SA, salicylic acid; UTR, untranslated region.

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nitrogen fixation, and uninfected cells and (4) senescence zone (zone 4).

Chitinases (EC 3.2.1.14) are ubiquitous enzymes of bacteria, fungi, animals and plants. They are able to cleave β-1,4-glycosidic bonds between *N*-acetylglucosamine residues of chitin, a structural polysaccharide of the cell wall of many fungi and of the exoskeleton of invertebrates (Cohen-Kupiec and Chet 1998). Plant chitinases have been implied in a wide variety of processes (reviewed by Kasprzewska 2003). The involvement of chitinases as an active or passive defence mechanism against pathogens has been largely reported and experimentally proved. In general, faster and higher induction and accumulation of chitinases correlates with resistance to pathogen attack (Gerhardt et al. 1997, Meier et al. 1993, Samac and Shah 1991). Chitinases are also involved in plant growth and development; they have been suggested to participate in cell elongation processes in *Arabidopsis thaliana* (Zhong et al. 2002) and in embryogenesis (Gerhardt et al. 1997, van Hengel et al. 1998, 2001). Chitinases are also known to act as antifreezing proteins during chilling and frost resistance (de los Reyes et al. 2001, Yeh et al. 2000, Yu and Griffith 2001) and as storage proteins (Peumans et al. 2002).

In legume nodules, chitinases have been postulated to participate in the plant autoregulatory mechanism that controls the intensity of root nodule formation and in regular nodule development by digesting bacterial lipo-chitooligosaccharides signal factors [Nod factors (NFs); Goormachtig et al. 1998, Staehelin et al. 1995, Vasse et al. 1993]. Furthermore, they have also been suggested to participate in nodule protection against external pathogens (Salzer et al. 2000, Staehelin et al. 1992). Far less information is available concerning the involvement of chitinases in actinorhizal symbioses. In fact, there is only one report on the expression of two chitinase encoding genes (EuNOD-CHT1 and EuNOD-CHT2) in root nodules of *Elaeagnus umbellata* that have been suggested to be involved in nodule development or protection against external pathogens (EuNOD-CHT1) and in a defence response against internal symbionts, external pathogens or both (EuNOD-CHT2) (Kim and An 2002). In any case the roles of chitinases in root nodule symbioses seem to be diverse. Here we report on the cloning and molecular characterization of a *Casuarina glauca* nodule-specific gene (*cgchi3*) encoding a putative class III chitinase.

Materials and methods

Plant and bacterial growth conditions

Casuarina glauca seeds were provided by the Australian Suppliers of Tree Seeds (Australian Tree Seed Centre, CSIRO Forestry and Forest Products, Kingston, Australia; www.ffp.csiro.au). Plants were grown and inoculated with *Frankia* Thr strain (Girgis et al. 1990) as described by Gherbi et al. (1997).

Wounding and salicylic acid treatments

For the wounding assays roots and leaves from control plants and nodules were mechanically wounded with a needle (Rushton et al. 2002) and harvested 1 h later. For the salicylic acid (SA) treatment the plant medium was replaced with a 5 m*M* SA solution (Pieterse et al. 1998) and the samples were harvested 6 h later.

Nucleic acid extraction

Casuarina glauca genomic DNA was extracted from young leaves using the DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. *Frankia* chromosomal DNA extraction was performed as previously described (Ribeiro et al. 1995). Total RNA extraction from *C. glauca* nodules, roots and leaves was performed using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany), following the modifications developed by Brunner et al. (2004).

Reverse transcription-PCR with degenerate primers

For the amplification of the complementary DNA (cDNA) fragment corresponding to class III chitinase, degenerate primers were designed based on conserved amino acid sequences of homologues from other plant species (Regalado et al. 2000, Salzer et al. 2000): 5′-GGGG-NCARAAYGGNAAYGAAGGG-3′ (forward) and 5′-CAI-GGNGGRTTRTTRTARAAYTG-3′ (reverse). Reverse transcription of 1 μg of nodule total RNA was performed with avian myeloblastosis virus (AMV) reverse transcriptase (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Thermal cycling was performed with a Trio-Thermoblock^{TM} cycler (Biometra, Göttingen, Germany) with the following cycling program: 1 cycle at 94°C for 2 min followed by 34 cycles at 94°C, 1.5 min—55°C, 1.5 min—72°C, 1.5 min and a final extension at 72°C for 5 min. The amplified product was cloned into pGEM-T Easy Vector System I (Promega, Madison, WI), following the instructions of the manufacturer, and sequenced.

Sequence analysis

Sequence reactions were performed at STAB VIDA (Oeiras, Portugal). Sequence similarity search was done at the National Center for Biotechnology Information

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(http:/www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tools, and sequence assembly was made using CAP3 Sequence Assembly Program (Huang and Madan 1999). The following Expert Protein Analysis System Proteomics Server (www.expasy.org) programs were also used: Translate (translation of nucleotide sequences to protein sequences), CLUSTAL W (multiple sequence alignment), ProtParam (protein physical and chemical parameters), PROSCAN (pattern and profile searches) and HMMTOP (prediction of transmembrane helices and topology of proteins).

Semiquantitative reverse transcription-PCR

cDNA was synthesised from total RNA of roots, leaves and nodules (control, wounded and SA treated) as described above. Specific primers were designed based on the partial cDNA sequence obtained after PCR with degenerated primers, using the program Primer 3 (Rozen and Skaletsky 2000), 5′-GGGGACAAAATGGGAACGA-AG-3′ (forward) and 5′-AAATGGACATTGAGGAGCT-GC-3′ (reverse). The optimal amounts of cDNA and the number of PCR cycles corresponding to the exponential phase of the reaction were determined. PCR was performed with a Trio-Thermoblock cycler (Biometra, Göttingen, Germany) with the program: 1 cycle at 94°C for 3 min followed by 35 cycles at 94°C, 30 s—60°C, 30 s—72°C for 30 and a final extension at 72°C for 5 min. Ubiquitin primers (Laplaze et al. 2002) were used as an internal control. As a control for genomic DNA contaminations, all reactions were performed in duplicate with the control sample lacking reverse transcriptase. As a control for unspecific amplifications, reactions with water instead of cDNA were included. Detailed procedures were as described in Chen et al. (2003). Ten microlitres of the amplified DNA samples was separated on a 1.2% agarose gel. All experiments were performed in triplicate.

Confirmation of PCR product identity by hybridisation

Fifteen microlitres of PCR product from semiquantitative PCR was separated on 1.2% agarose gels and transferred to nylon membranes (Roche, Mannheim, Germany) according to Sambrook et al. (1989). Membranes were hybridized with a digoxigenin (DIG)-labelled *cgchi3* probe (DIG High prime DNA labelling and detection starter kit II, Roche, Mannheim, Germany) according to the manufacturer's instructions.

PCR on genomic DNA

PCR on *C. glauca* and *Frankia* genomic DNA (100 ng each) was performed using the program described above.

Southern blot analysis of genomic DNA

Southern blots containing 15 μg of *C. glauca* DNA digested with restriction enzymes *Hin*dIII and *Bam*HI/*Pst*I, respectively, were prepared as described by Sambrook et al. (1989). Membranes were hybridized as described above.

Rapid amplification of cDNA ends

Rapid amplification of cDNA ends (RACE)-PCR was done with BD SMART Race cDNA amplification kit (Clontech, Palo Alto, CA), following the manufacturer's instructions using gene-specific primers 5′-GTAACTCCCAGCCCCT-CCCCCAAG-3′ and 5′-GAACAAGTCTCTGCCAAGGTG-3′ (nested) (5′ end) and 5′-CTTGGGGGAGGGGCTGGGAGT-TAC-3′ (3′ end). RACE products were cloned into pGEM-T Easy Vector System I (Promega, Madison, WI) and sequenced as described above.

In situ hybridisation

Nodules were fixed, embedded and sectioned as described by Ribeiro et al. (1995). Hybridisation pretreatment, washing and detection of DIG-labelled probes were performed essentially as described by Jackson (1991) and on the web page referenced in Long et al. (1996), except that no washes in 0.2 × saline–sodium citrate (SSC) were performed before RNase treatment, and after washes in RNase buffer slides were washed first for 30 min in 2 × SSC, and then for 30 min at 56°C in 0.2 × SSC in a shaking water bath. Hybridisation conditions were as described by Ribeiro et al. (1995).

For in vitro transcription, plasmids were linearized and labelled RNA was produced using RNA polymerase in the presence of DIG-labelled uridine 5′-triphosphate (Roche, Mannheim, Germany) according to the instructions of the manufacturer. *Frankia nifH* antisense RNA was prepared as described by Ribeiro et al. (1995). pCgChi3 was linearized with *Sal*I and antisense RNA was produced using T7 RNA polymerase; for the production of sense RNA, pCgChi3 was linearized with *Bam*HI and in vitro transcribed using T3 RNA polymerase. After in vitro transcription, the template DNA was removed via treatment with RNase-free DNase, and the non-incorporated nucleotides were removed via MicroSpin™ S-300 HR columns (Amersham Pharmacia, Piscataway, NJ).

Results

Cloning and sequencing analysis of *cgchi3* cDNA

Cgchi3 was first isolated as a short cDNA fragment (477 bp) after reverse transcription-PCR (RT-PCR) on total RNA

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from *C. glauca* nodules with degenerate primers. Two fragments of 160 and 493 bp corresponding to the 5′ and 3′ region of *cgchi3*, respectively, have been amplified by RACE. The *cgchi3* full-size cDNA was 1130 bp in length, consisting of a 60-bp 5′-untranslated region (UTR), a complete open reading frame (ORF) of 894-bp encoding a polypeptide of 298 amino acids, followed by a 3′-UTR of 173 bp (GenBank accession number EF134410; data not shown). The putative protein encoded by *cgchi3* showed 78 % identity with other plant class III chitinases (Fig. 1). Analysis of the CGCHI3 ORF with PROSCAN revealed several potential phosphorylation sites: (1) five protein kinase C recognition sites: [ST]-x-[RK] where S or T is the phosphorylation residue (amino acids: 114 to 116—SSK; 135 to 137—SSR, 155 to 157—TNK, 236 to 238—TNK and 271 to 273—SSK); (2) five casein kinase II recognition sites: [ST]-xx-[DE] where S or T is the phosphorylation site (amino acids: 40 to 43—TLAE, 60 to 63—TFGD, 114 to 117—SSKD, 189 to 192 SSGE and 216 to 219—SSGE) and (3) two tyrosine kinase recognition sites: [RK]-xx-[DE]-xxx-Y or [RK]-xxx-[DE]-xx-Y where Y is the phosphorylation site (amino acids: 157 to 165—KHWDELARY and 282 to 290—KYYDDQSGY). CGCHI3 also possesses the glycosyl hydrolase family 18 active site: [LIVMFY]-[DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-x-E where E is the active site residue (amino acids: 144 to 152—LDGIDFDIE).

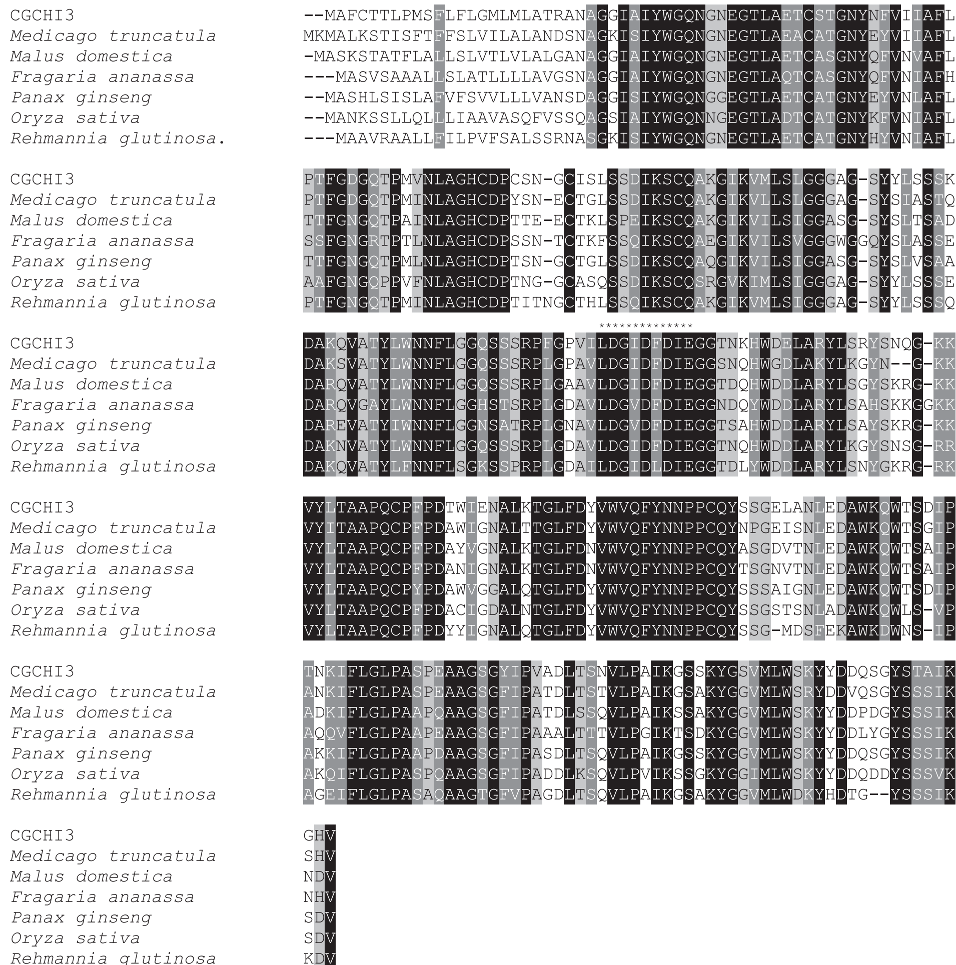

Fig. 1. Multiple sequence alignment of the deduced amino acid sequence of CGCHI3 with plant class III chitinases. Amino acid residues that are perfectly conserved among all sequences are highlighted in black, amino acid residues that are conserved are highlighted in dark grey and amino acid residues that are semiconserved are highlighted in light grey. Dashes represent gaps introduced to maximize alignment. The active site of family 18 glycosyl hydrolase is indicated by asterisks (*). The protein accession numbers for the retrieval of these chitinases sequences from the National Center for Biotechnology Information database are *Medicago truncatula*—AAQ21404, *Malus domestica*—AAG25709, *Frangaria ananassa*—AAD22114, *Panax ginseng*—ABF82271, *Oryza sativa*—XP_477539 and *Rehmannia glutinosa*—AAO47731, *Casuarina glauca*—EF134410.

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PCR on genomic DNA and Southern blot analysis

PCR on *C. glauca* and *Frankia* genomic DNA confirmed that *cgchi3* was indeed from plant origin. Using gene-specific primers a PCR product (477 bp) was amplified only when *C. glauca* DNA was used as template (Fig. 2). The pattern of hybridisation bands on a Southern blot suggests that *cgchi3* is encoded by a single gene (Fig. 3).

Expression analysis of *cgchi3*

Expression levels of *cgchi3* were examined in roots, leaves and nodules by semiquantitative RT-PCR (Fig. 4). For all types of organs, control, wounded and SA-treated samples were analysed. *Cgchi3* was found to be expressed specifically in nodules; no transcripts were detected in roots and leaves. Expression levels of *cgchi3* in nodules were enhanced by SA, while expression was abolished in response to wounding.

In situ hybridisation studies were performed to examine in which zone of the nodule *cgchi3* was expressed. Longitudinal nodule sections and cross sections of nodule lobes were hybridized with DIG-labelled antisense and sense *cgchi3* RNA probes. In younger nodules (ca. five weeks after infection), *cgchi3* expression was detected in the meristem and in the uninfected and infected cortical cells in the younger part of the nodule lobe (Fig. 5A). High expression levels were also detected in the phloem and pericycle of the vascular bundle (Fig. 5A). In older nodules (ca. eight weeks after infection), *cgchi3* expression was found in the phloem and pericycle of the vascular bundle and in many, though not all infected cells (Fig. 5E). Nitrogen-fixing infected cells were identified based on expression of the bacterial nitrogenase structural gene *nifH* (Fig. 5C).

Fig. 3. Southern blot analysis. H, *Casuarina glauca* genomic DNA digested with *Hin*dIII; B/P, *C. glauca* genomic DNA digested with *Bam*HI/*Pst*I; M, molecular marker (1-kb ladder, Invitrogen, Carlsbad, CA).

Discussion

In an attempt to contribute to the clarification of the role of chitinases during root nodule symbioses, we have cloned a cDNA corresponding to a class III chitinase gene (*cgchi3*) from actinorhizal nodules of *C. glauca*. *Cgchi3* is an early nodulin (*ENOD*) gene whose expression is absolutely nodule specific. Its expression is further enhanced by SA and completely repressed by wounding. Based on the results obtained, several hypotheses on the role of *cgchi3* during *C. glauca* nodulation might be brought forward.

In legumes, chitinases have received particular attention because they can degrade rhizobial NFs thus

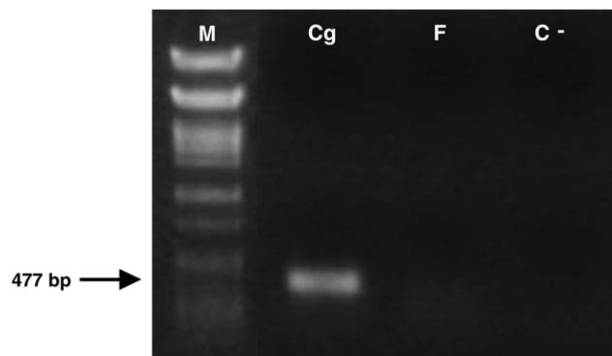

Fig. 2. PCR on genomic DNA. M, molecular weight marker (λ DNA digested with the enzyme *Pst*I); Cg, *Casuarina glauca* genomic DNA; F, *Frankia* genomic DNA and C−, negative control.

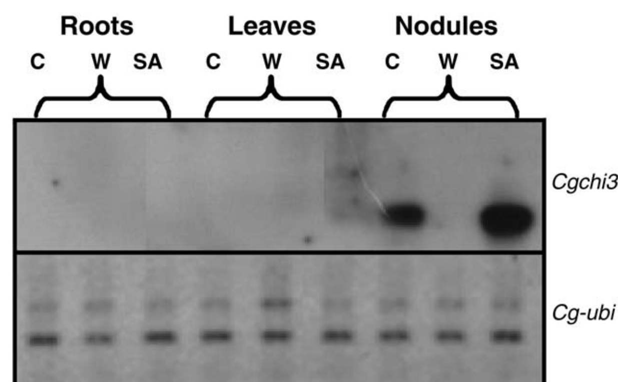

Fig. 4. Expression analysis of *cgchi3* in roots, leaves and nodules and upon external stimuli. C, control; W, wounded; SA, SA-treated.

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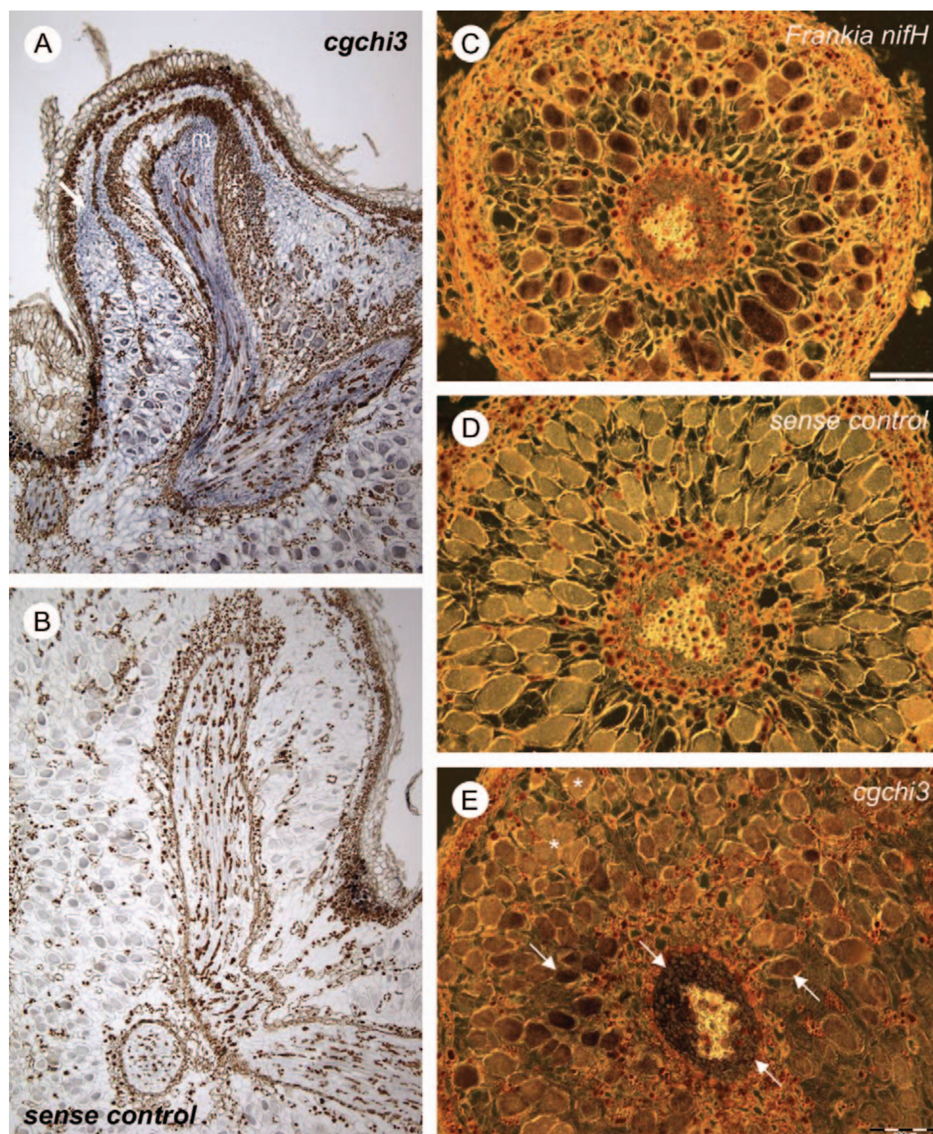

Fig. 5. In situ hybridisation of nodule sections with *Casuarina glauca cgchi3* antisense RNA (A, E), *C. glauca cgchi3* sense RNA (B, D) and *Frankia nifH* antisense RNA (C). Panels A and B were photographed using bright field optics; panels C to E were photographed in dark field to improve the visibility of plant structures. Purple/brownish precipitate marks the places of hybridisation. A comparison of (C) and (E) shows that in older nodules, *cgchi3* is expressed at highest levels in the phloem and pericycle of the vascular bundle and in many infected cells (arrows), thought not in all (asterisks). (A) In younger nodules, *cgchi3* expression is also found in the uninfected cortical cells in the younger part of the nodule lobe (arrow) and in the meristem of the nodule lobe (m). The size bar denotes 100 μm.

controlling the frequency of root nodule formation (Goormachtig et al. 1998, Heidstra et al. 1994, Staehelin et al. 1995). The putative protein encoded by *cgchi3* contains in its active site, typical for the glycosyl hydrolase family 18, two amino acid residues, D and E, that are a prerequisite for chitinolytic activity (Anderson et al. 1997, Watanabe et al. 1993), indicating that CGCHI3 really has chitinolytic activity. So far, no equivalents of rhizobial NFs have yet been identified in symbioses between *Frankia* and actinorhizal plants. The presence of a root hair-deforming factor was also recognized in the *Alnus glutinosa*–*Frankia* system (van Ghelue et al. 1997) but *Frankia* factors seem to be chemically different from rhizobial NFs, suggesting that nodulation of actinorhizal plants is not under feedback regulation through the degradation of NFs by chitinases (Cérémonie et al. 1998, 1999). This information, together with the fact that high levels of *cgchi3* expression are found in many cells that are far from infected cells where *Frankia* signal factors might be produced, seems to

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indicate that CGCHI3 is not involved in the degradation or deactivation of *Frankia* factors, whatever their chemical nature may be.

Chitinases have also been implied in growth and development processes (reviewed by Kasprzewska 2003). In *Daucus carota*, chitinases are able to process ArabinoGalactan Proteins (AGPs), which in turn seem to be involved in the regulation of cell differentiation (van Hengel et al. 2001). The fact that *cgchi3* transcripts are present in cortical cells at all stages of differentiation (up to mature infected cells) might suggest that CGCHI3 participates in nodule development, may be by processing (a) substance(s), like AGPs, that are known to be part of the plant matrix surrounding the bacteria (Berry et al. 2002). A developmental function of CGCHI3 is further supported by the fact that wounding (and the consequent stop of nodule growth) completely abolished *cgchi3* expression. However, processing of a compound of the perisymbiont matrix cannot be the only role of CGCHI3 because high levels of *cgchi3* transcripts are also found not only in mature infected cells but also in the nodule vasculature, uninfected cortical cells and the nodule meristem.

In *Musa* spp. a protein homologous to class III chitinases serves as a storage protein and is used as a source of amino acids for the synthesis of ripening-related proteins (Peumans et al. 2002). Based on the pattern of *cgchi3* transcript accumulation in *C. glauca* nodules, it might also be suggested that CGCHI3 is a developmentally regulated storage protein that participates in nitrogen and carbon metabolism. However, the fact that higher levels of *cgchi3* expression are detected in younger nodules than in older ones, together with the fact that *cgchi3* expression is induced by SA, weakens this hypothesis.

Because *cgchi3* expression is enhanced after SA treatment we cannot exclude that this gene is part of the nodule defence response mediated by SA and that it is involved in nodule protection. However, given the high expression levels of *cgchi3* in non-SA-treated nodules this is unlikely to be its only function.

In general, wounding and SA are known to induce the transcription of chitinase genes (Kasprzewska 2003). However, in contrary to SA, wounding abolished *cgchi3* transcription. Because wounding activates the jasmonic acid (JA)-signalling pathway (Baldwin et al. 1994, 1997, Creelman and Mullet 1997), it seems likely that *cgchi3* expression is repressed by JA. The roles of JA and SA during root nodule symbioses are not clear but in many legumes the accumulation of these signal substances has a negative effect on nodulation (Nakagawa and Kawaguchi 2006, Sato et al. 2002, Sun et al. 2006). Sato et al. (2002) and Nakagawa and Kawaguchi (2006) have, respectively, suggested that JA and SA participate in the autoregulatory process that controls nodule number in legumes. It is possible that they act by controlling the activation of defence responses (Ferguson and Mathesius 2003, Stacey et al. 2006). Although our data do not allow to draw any conclusions on this issue, it would be interesting to analyse in depth the effects of JA and SA on actinorhizal nodule development and how these factors modulate *cgchi3* expression in the course of nodulation.

In conclusion, we have cloned and characterized at the molecular level a gene encoding a class III chitinase, *cgchi3*, that seems to have a specific function during *C. glauca* nodulation by *Frankia*. Although further studies are required to ascertain the function of this chitinase, we speculate that it is involved in nodule development.

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