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SHORT NOTE

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Differential expression of two class III chitinases in two types of roots of *Quercus robur* during pre-mycorrhizal interactions with *Piloderma croceum*

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Abstract Expression of two plant chitinase genes, representing members of class III chitinases, was studied in *Quercus robur* roots during interactions in a pre-mycorrhizal stage with the ectomycorrhizal fungus *Piloderma croceum*. Chitinase gene expression was compared in lateral roots destined to form ectomycorrhiza, and in principal roots that are not directly involved in mycorrhizal interactions. The transcript level of the first chitinase (*QrchitIII-1*) was upregulated in lateral roots, whereas no significant differential expression was observed in principal roots. The second chitinase (*QrchitIII-2*) was regulated neither in lateral nor in principal roots in presence of the fungus. Because *P. croceum* did not induce significant chitinase responses in principal roots, the enhanced expression of *QrchitIII-1* in lateral roots after inoculation may be related to some steps in symbiosis ontogenesis.

Keywords Ectomycorrhiza · Class III chitinases · Gene expression · *Piloderma croceum* · *Quercus robur*

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Introduction

Seeds germinate and plants grow and die in interaction with a large spectrum of microorganisms. Chitinases are key enzymes involved in plant-microbe interactions and are grouped in the pathogen-related protein type three families (PR-3) (Selitrennikoff 2001). Five classes of plant chitinases have been proposed based on their peptidic sequences, conserved domains and specific activities (Selitrennikoff 2001). Chitinases catalyse the hydrolysis of chitin, an insoluble linear homo-polymer of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues (Collinge et al. 1993). Because chitin is a primary structural component of the wall of all true fungi, chitinases are considered to play a major role during pathogenic plant-fungal interactions (Collinge et al. 1993; Selitrennikoff 2001; Bravo et al. 2003). However, chitinase coding genes have been found to be over-expressed and/or chitinase activities enhanced not only during fungal (Albrecht et al. 1994; Pozo et al. 1998; Davis et al. 2002), bacterial (Gerhardt et al. 1997) or viral (Balsalobre et al. 1997; Burketova et al. 2003) pathogenic infections, but also during symbiotic interactions with *Rhizobium* (Goormachtig et al. 1998; Kim and Sun 2002) or mycorrhizal fungi (Albrecht et al. 1994; Dumas-Gaudot et al. 1994; Hodge et al. 1996; Salzer et al. 2000; Bestel-Corre et al. 2002). Class III chitinases have been reported to be specifically transcribed during arbuscular mycorrhiza interactions (Salzer et al. 2000), but only chitinase enzyme activities have been assessed in the ectomycorrhizal symbiosis (Sauter and Hager 1989; Albrecht et al. 1994).

In the present work, we used a culture system of micro-cuttings of *Quercus robur* and the ectomycorrhizal fungus

Piloderma croceum which is characterised by a long pre-mycorrhizal stage during which the fungus stimulates growth of the lateral root system but not of the principal roots (Herrmann et al. 1998). Physiological responses (Herrmann et al. 2004) and specific gene regulation (Krüger et al. 2004) have been shown to occur in the plant during this pre-mycorrhizal stage. Here, we targeted class III chitinase gene expression during the pre-mycorrhizal stage between *Q. robur* and *P. croceum*, and compared chitinase gene regulation in lateral roots where the symbiosis becomes established, and in principal roots which are not directly involved in mycorrhizal interactions with the fungus. The roles of class III chitinases during the ectomycorrhizal symbiosis are discussed in the light of the observed gene expression profiles.

Materials and methods

Biological material

The pre-mycorrhizal interaction between micro-propagated *Quercus robur* L. (clone DF 159) and *Piloderma croceum* J. Erikss. & Hjortst. (strain 729 from the “TUMY”, University of Tübingen, Germany) was established as described by Herrmann et al. (1998). Briefly, oak plants were micro-propagated and rooted aseptically, then transferred into a mycorrhization system consisting in Petri dishes (diameter 90 mm) with MMN medium with 1:10 nitrogen and phosphorus in which roots develop aseptically and two dimensionally, whilst the shoot grows outside of the dish. Each mycorrhization system was placed inside a larger Petri dish (diameter 140 mm) in which humidity was regulated by moistened paper to prevent shoot desiccation (Krüger et al. 2004). Control non-inoculated plants and inoculated plants were cultivated vertically in growth chambers according to Herrmann et al. (1998) and Krüger et al. (2004).

Root harvesting was performed after 4 weeks of culture, at the end of the first root flush, corresponding to the beginning of the apical bud outburst (Herrmann et al. 1998). At this stage, contact between roots and inoculum was highly limited and no mycorrhiza was developed. Newly formed lateral roots and principal roots were frozen in liquid nitrogen before RNA extraction. Three replicates of independent biological experiments were performed for pre-mycorrhizal analysis. Per replicate, about 20 plants were used for each condition (control or inoculated) to obtain enough transcripts.

Cloning and sequencing of class III chitinases from *Q. robur* DNA

Two degenerate primers (Table 1) were designed for plant class III chitinases based on conserved amino acid motifs. Primers used in this study were synthesised by Invitrogen Life Technology (Karlsruhe, Germany). Oak genomic DNA was isolated from frozen material after grinding using the Dneasy Plant Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. For polymerase chain reaction (PCR) amplification, 1 μg of

Table 1 Degenerate primers[a] used for polymerase chain reaction (PCR) on genomic DNA from *Quercus robur* and specific primers for *QrchitIII-1* and *QrchitIII-2* reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR analysis

Primer types	Oligonucleotide sequences	Fragment sizes (bp)	Database organism matches	*E*-value	GenBank Accession number	Primer specificity
Degenerate primers[a] for class III chitinases	5′ GGN GAY GCN GTN YTN GAY GG 3′	198 to 201	–	–	–	
	5′ YTG NAC CCA NAC GTA RTC RA 3′					
Specific primers for *QrchitIII-1*	5′ GAC TTT GAT ATT GAA GGA GG 3′	160	*Dioscorea oppositifolia*	9e-27	AJ879088	*Q.r. P.c.*
	5′ ATA GAC CTG TCT TAA GGG CAG C 3′					
Specific primers for *QrchitIII-2*	5′ GAT TTT GGC ATT GAG ATT GG 3′	157	*Cicer arietinum*	2e-16	AJ879089	*Q.r. P.c.*
	5′ ACA AGC CGG TTT TGA TAT CGG 3′					

The last column shows primer specificities to plant, with amplifications with cDNA of *Q. robur* (*Q.r.*) and no amplification with cDNA of *P. croceum* (*P.c.*)

[a]In degenerate primers *N* A, C, T or G, *Y* C or T, *R* A or G

genomic DNA was added to a 50 µl reaction mixture containing 5 µl of 10× *Taq* buffer with $MgCl_2$ (Q-BIOgene, Heidelberg, Germany), 4 µl of dNTPs (2 mM each) (MBI Fermentas, St Leon-Rot, Germany), 1 µl of each primer (60 µM) and 0.2 µl of *Taq* DNA polymerase (Q-BIOgene, Heidelberg, Germany). PCRs were run on a Primus 96 plus thermocycler (MWG-Biotech, England) with an initial cycle of denaturation (5 min at 94°C) followed by 30 cycles with denaturation (1 min 94°C), annealing (30 s at 50°C) and elongation (2 min at 72°C) and by a final elongation (10 min at 72°C).

PCR products were directly cloned into a pCR4-TOPO vector, using the TOPO TA Cloning kit for sequencing (Invitrogen Life Technologies, Karlsruhe, Germany) according to the manufacturers' instructions. The sequencing reactions were run on an automated multi-capillary DNA sequencer, ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator v.3.1. Ready Reaction Cycle Sequencing kit (Applied Biosystems) as recommended by the manufacturer. Genomic class III chitinase gene sequences obtained from *Q. robur* have been deposited at EMBL (see Table 1 for the accession numbers).

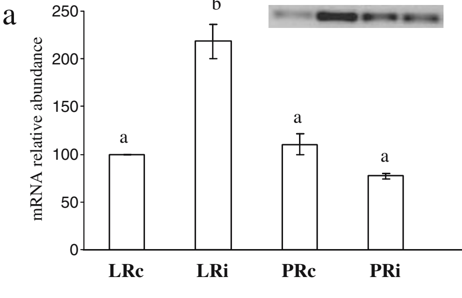
Extraction and analysis of RNA

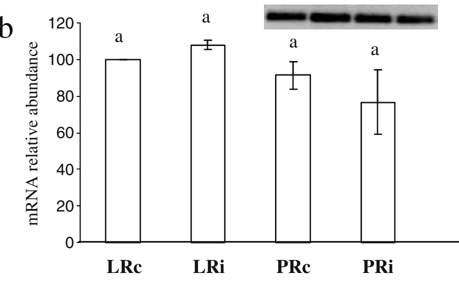
RNA extractions from control and inoculated roots of *Q. robur*, and from *P. croceum* were performed according to Wang et al. (2000). cDNA was synthesised from 1µg of total RNA using the BD SMART PCR cDNA Synthesis Kit (Clontech, Heidelberg, Germany) as recommended by the manufacturer. To perform expression analysis, specific primer sets were designed for the two classes of III chitinases isolated from oak (Table 1). Reverse transcription polymerase chain reaction (RT-PCR) was performed on a Primus 96 plus thermocycler (MWG-Biotech, England) under the following conditions: an initial cycle of denaturation (2 min at 94°C), followed by 20 cycles with denaturation (30 s at 94°C), annealing (30 s at 55°C) and elongation (45 s at 72°C) and by a final elongation (10 min at 72°C). Expression of 26S ribosomal RNA was used as control (Fig. 1). Specific primers used to amplify the 26S region under the same PCR conditions were: 5′ AAC GCT TGG CTG CCA CAA GCC 3′ and 5′ GAC TCT CTT AAG GTA GCC AAA TGC C 3′. From each PCR reaction, 5 µl was loaded onto a 2% agarose gel. To confirm primer specificities, amplificats were extracted from gels with QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany), cloned and sequenced as previously described. PCR with *P. croceum* cDNA was performed to eliminate the possibility of fungal class III chitinase amplification (Table 1).

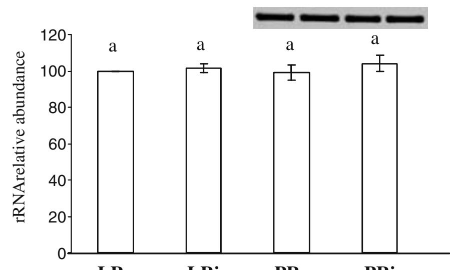
Three technical repetitions on the three independent biological replicates were performed by quantitative real-time PCR on 30 ng of cDNA using iQ SYBR Green Super mix (Bio-Rad Laboratories, Hercules, CA, USA) on iCycler Thermal Cycler (Bio-Rad) as follows: initial denaturation (2 min at 95°C), 40 cycles (30 s at 95°C, 40 s at 55°C, 45 s at 72°C), final extension (10 min 72°C). A melting curve analysis validated primer gene specificity. Differences in expression between treatments were calculated according to the "Delta-delta method" (Pfaffl 2001) using the 26S rRNA as reference.

Data acquisition and statistical analysis

Expression levels were quantified using ImageQuant version 5.0 Software (Molecular Dynamics, Amersham Biosciences). To allow comparison between different experiments, the value of 100 was given to the level of expression in lateral roots of control plants. Data were







d

	LRi/LRc	PRi/PRc
QrchitIII-1	2.84	-1.32
QrchitIII-2	1.22	-1.19
26S rRNA	1.07	1.05

Fig. 1 Expression of *QrchitIII-1* (**a**) and *QrchitIII-2* (**b**) in lateral roots of control (LRc) and inoculated plants (LRi) in principal roots of control (PRc) and inoculated plants (PRi) during *Q. robur–P. croceum* interaction. The transcript levels were standardized to the non-regulated 26S rRNA (**c**). *Bars* indicate standard errors of means. *Common letters* indicate non-significant differences between tissues. For each gene, one reverse transcription polymerase chain reaction (RT-PCR) gel is presented as example in the same lecture order (LRc, LRi, PRc and PRi). Fold changes obtained by quantitative real-time RT-PCR analysis of three technical replicates performed on the three biological samples are presented in (**d**)

submitted to multiple comparisons (Student test, one way ANOVA test, $P<0.05$) with SigmaStat Version 2.03 software.

Results and discussion

Two partial oak DNA sequences, *QrchitIII-1* (accession n° AJ879088) and *QrchitIII-2* (accession n° AJ879089) with high similarities to other plant class III chitinases (Table 1) were isolated and their transcript levels analysed at the pre-mycorrhizal stage during the interaction with *P. croceum* (Fig. 1). Chitinase gene expression was monitored by RT-PCR analysis (Fig. 1a,b) and regulation estimated by quantitative real-time PCR (Fig. 1d). The transcript level of *QrchitIII-1* was upregulated 2.84-fold in lateral roots (Fig. 1d), whereas no significant regulation was observed in principal roots (Fig. 1a,d). The second chitinase, *QrchitIII-2* was regulated neither in lateral nor in principal roots in presence of the fungus (Fig. 1b,d). Because *P. croceum* did not induce a significant chitinase response in principal roots, the strong expression of *QrchitIII-1* in lateral roots in response to inoculation might be related to some steps in symbiosis ontogenesis.

The role of *QrchitIII-1* during ectomycorrhizal symbiosis establishment still has to be elucidated. According to the literature, this enzyme could act in one of two ways. Class III chitinases are expressed in plants during wounding (Rojas-Herrera and Loyola-Vargas 2002) and during aggression by pathogenic microorganisms (Regalado et al. 2000). Recently, Le Quéré et al. (2005) observed an up-regulated chitinase during mantel and Hartig net development in the birch-*Paxillus* interaction. Therefore *Q. robur* might express such a gene as *QrchitIII-1* to modulate the hyphal growth of *P. croceum* before symbiotic structure establishment. Alternatively, *QrchitIII-1* might be involved in modulating elicitor release by *P. croceum*. Elicitors are signal molecules which can be released by symbiotic or pathogenic fungi and which induce defence responses in plants (Salzer et al. 1996; Kästner et al. 1998). The over-expression of a class III chitinase gene was detected in the presence of such elicitors (Busam et al. 1997). It has been suggested that during symbiotic ectomycorrhizal interactions, stimulation of chitinase may be related to the suppression of plant defence reactions through the cleavage of chitinous elicitors produced by the fungal symbiont (Salzer et al. 1997a,b). Goormachtig et al. (1998) have demonstrated that the protein encoded by the cDNA clone *Srchi13*, an homolog to acidic class III chitinase genes, exhibits Nod-factor (the rhizobial elicitor) degrading activity. The lateral root specific over-expression of *QrchitIII-1* (Fig. 1a) during the presymbiotic stage of the *Q. robur–P. croceum* interaction could limit the elicitor presence around this root type and authorise hyphal penetration by restricting plant defence activation.

According to our results, a member of the class III chitinase family may be associated with initial steps of ectomycorrhiza establishment in *Q. robur* because expression of this gene is modulated only in the root type where ectomycorrhiza formation is likely to occur. It remains to be enlightened whether class III chitinase over-expression corresponds to plant defence response or to elicitor modulation. The absence of regulation of *QrchitIII-2* (Fig. 1b) indicates that only a selective chitinase gene regulation is activated by the fungus.

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References

Albrecht C, Asselin A, Piché Y, Lapeyrie F (1994) Chitinase activities are induced in *Eucalyptus globulus* roots by ectomycorrhizal or pathogenic fungi, during early colonization. Physiol Plant 91:104–110

Balsalobre JM, Mas P, Sanchez–Pina MA, Pallas V (1997) Spatial distribution of acidic chitinases and their messenger RNAs in tobacco plants infected with cherry leaf roll virus. Mol Plant–Microb Interact 10:784–788

Bestel-Corre G, Dumas-Gaudot E, Gianinazzi-Pearson V, Gianinazzi S (2002) Mycorrhiza-related chitinase and chitosanase activity isoforms in *Medicago truncatula* Gaertn. Symbiosis 32:173–194

Bravo JM, Campo S, Murillo I, Coca M, San Segundo B (2003) Fungus- and wound-induced accumulation of mRNA containing a class II chitinase of the pathogenesis-related protein (PR-4) family of maize. Plant Mol Biol 52:745–759

Burketova L, Stillerova K, Feltlova M (2003) Immunohistological localization of chitinase and β-1,3-glucanase in rhizomania-diseased and benzothiadiazole treated sugar beet roots. Physiol Mol Plant Pathol 63:47–54

Busam G, Kassemeyer HH, Matern U (1997) Differential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators or fungal challenge. Plant Physiol 115:1029–1038

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. Plant J 3:31–40

Davis JM, Wu H, Cooke JEK, Reed JM, Luce KS, Michler CH (2002) Pathogen challenge, salicylic acid, and jasmonic acid regulate expression of chitinase gene homologs in pine. Mol Plant–Microb Interact 15:380–387

Dumas-Gaudot E, Asselin A, Gianinazzi-Pearson V, Gollotte A, Gianinazzi S (1994) Chitinase isoforms in roots of various pea genotypes infected with arbuscular mycorrhizal fungi. Plant Sci 99:27–37

Gerhardt LBD, Sachetto-Martins G, Contarini MG, Sandroni M, Ferreira RD, de Lima VM, Cordeiro MC, de Oliveira DE, Margis-Pinheiro M (1997) *Arabidopsis thaliana* class IV chitinase is early induced during the interaction with *Xanthomonas campestris*. FEBS Lett 419:69–75

Goormachtig S, Lievens S, Van de Velde W, Von Montagu M, Holsters M (1998) Srchi13, a novel early nodulin from *Sesbania rostrata*, is related to acidic class III chitinase. Plant Cell 10:905–915

Herrmann S, Munch JC, Buscot F (1998) A gnotobiotic culture system with oak microcuttings to study specific effects of mycobionts on plant morphology before, and in the early phase of, ectomycorrhiza formation by *Paxillus involutus* and *Piloderma croceum*. New Phytol 138:203–212

Herrmann S, Oelmüller R, Buscot F (2004) Manipulation of the onset of ectomycorrhiza formation by indole-3-acetic acid, activated charcoal or relative humidity in the association between oak microcuttings and *Piloderma croceum*: influence on plant development and photosynthesis. J Plant Physiol 161:509–517

Hodge A, Alexander IJ, Gooday GW, Williamson FA (1996) Localization of chitinolytic activities in *Fagus sylvatica* mycorrhizas. Mycorrhiza 6:181–187

Kästner B, Tenhaken R, Kauss H (1998) Chitinase in cucumber hypocotyls is induced by germinating fungal spores and by fungal elicitor in synergism with inducers of acquired resistance. Plant J 13:447–454

Kim HB, Sun AC (2002) Differential expression patterns of an acidic chitinase and a basic chitinase in the root nodule of *Elaeagnus umbellata*. Mol Plant–Microb Interact 15:209–215

Krüger A, Peškan-Berhöfer T, Frettinger P, Herrmann S, Buscot F, Oelmüller R (2004) Identification of premycorrhiza-related plant genes in the association between *Quercus robur* and *Piloderma croceum*. New Phytol 163:149–157

Le Quéré A, Wright DP, Söderström B, Tunlid A, Johansson T (2005) Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) Fr. Mol Plant–Microb Interact 18:659–673

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:2002–2007

Pozo MJ, Azcón-Aguilar C, Dumas-Gaudot E, Barea JM (1998) Chitosanase and chitinase activities in tomato roots during interactions with arbuscular mycorrhizal fungi or *Phytophthora parasitica*. J Exp Bot 49:1729–1739

Regalado AP, Pinheiro C, Vidal S, Chaves I, Ricardo CPP, Rodrigues-Pousada C (2000) The *Lupinus albus* class-III chitinase gene, *IF3*, is constitutively expressed in vegetative organs and developing seeds. Planta 210:543–550

Rojas-Herrera R, Loyola-Vargas VM (2002) Induction of a class III acidic chitinase in foliar explants of *Coffea arabica* L. during somatic embryogenesis and wounding. Plant Sci 163:705–711

Salzer P, Hebe G, Reith A, Zitterell-Haid B, Stransky H, Gaschler K, Hager A (1996) Rapid reactions of spruce cells to elicitors released from the ectomycorrhizal fungus *Hebeloma crustuliniforme*, and inactivation of these elicitors by extracellular spruce cell enzymes. Planta 198:118–126

Salzer P, Hebe G, Hager A (1997a) Cleavage of chitinous elicitors from the ectomycorrhizal fungus *Hebeloma crustuliniforme* by host chitinases prevents induction of K^+ and Cl^- release, extracellular alkalinisation and H_2O_2 synthesis of *Picea abies* cells. Planta 203:470–479

Salzer P, Hübner B, Sirrenberg A, Hager A (1997b) Differential effect of purified spruce chitinases and ß-1,3-glucanases on the activity of elicitors from ectomycorrhizal fungi. Plant Physiol 114:957–968

Salzer P, Bonanomi A, Beyer K, Vögeli-Lange R, Aeschbacher RA, Lange J, Wiemken A, Kim D, Cook DR, Boller T (2000) Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. Mol Plant–Microb Interact 13:763–777

Sauter M, Hager A (1989) The mycorrhizal fungus *Amanita muscaria* induces chitinase activity in roots and in suspension cultured cells of its host *Picea abies*. Planta 179:61–66

Selitrennikoff CP (2001) Antifungal proteins. Appl Environ Microbiol 67:2883–2894

Wang SH, Hunter W, Plant A (2000) Isolation and purification of functional total RNA from woody branches and needles of Sitka and white spruce. Biotechniques 28:292–296