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Characterization of four defense-related genes up-regulated in root nodules of *Casuarina glauca*

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Abstract Actinorhizal plants are capable of high rates of nitrogen fixation, due to their capacity to establish a root-nodule symbiosis with N_2-fixing actinomycetes of the genus *Frankia.* Nodulation is an ontogenic process which requires a sequence of highly coordinated events. One of these mechanisms is the induction of defense-related events, whose precise role during nodulation is largely unknown. In order to contribute to the clarification of the involvement of defense-related genes during actinorhizal root-nodule symbiosis, we have analysed the differential expression of several genes with putative defense-related functions in *Casuarina glauca* nodules *versus* non-inoculated roots. Four genes encoding a chitinase (*CgChi1*), a glutathione *S*-transferase (*CgGst*), a hairpin-inducible

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protein (*CgHin1*) and a peroxidase (*CgPox4*) were found to be up-regulated in mature nodules compared to roots. In order to find out to which extend were the encoded proteins involved in nodule protection, development or both, gene regulation studies in response to SA and wounding as well as phylogenetic analysis of the protein sequences were performed. These were further characterized through expression studies after SA-treatment and wounding, and by phylogenetic analysis. We suggest that *CgChi1* and *CgGst* are involved in defense or microsymbiont control and *CgPox4* is involved in nodule development. For *CgHin1* the question "defense, development or both" remains open.

Keywords Actinorhizal symbiosis · *Casuarina glauca* · *Frankia* · Defense · Chitinases · NHL · GST · Peroxidases

1 Introduction

The ability of certain plants to establish symbiotic relationships with soil bacteria for the purpose of fixing atmospheric nitrogen into ammonia has a tremendous impact on natural and agricultural ecosystems. In two groups of nitrogen fixing symbiotic interactions, the prokaryotic partners are soil bacteria—rhizobia in legume symbioses, and *Frankia* strains in actinorhizal symbioses—and N_2 fixation takes place in a newly formed plant organ, the root nodule (reviewed by Pawlowski and Bisseling 1996). In total, actinorhizal symbioses have become increasingly important as climate changes threaten to remake the global landscape over the next decades. Most actinorhizal plants (a group of woody plants from eight different families) are capable of high rates of nitrogen fixation, due to their capacity to establish root nodule symbiosis with N_2-fixing *Frankia* strains. These plants are able to grow in poor and disturbed soils and are important elements in plant communities worldwide. They have a great capability for adaptation and can be used for fuel wood production, agro-forestry, and land reclamation.

Root nodule formation is an intricate and complex process that must be tightly regulated. In legumes, the picture emerging from recent research indicates that similar mechanisms are used by the plant to recognize and accommodate pathogens and symbiotic microbes, and it has been proposed that legume nodulation evolved from a pathogenic interaction (Sprent 2007). Events like localized cell death, production of antimicrobial compounds, as well as the induction of host stress and defense-related genes/proteins have been reported by several groups (*e.g.* Spaink 1995; Samac and Graham 2007). Additionally, transcriptome analysis indicates that in legume nodules, the percentage of genes belonging to the cell defense/resistance/rescue category is 5% on average (*e.g.* Ramírez et al. 2005; Lohar et al. 2006), thus indicating a significant role for defense signalling in the early events leading to nodulation.

Far less is known about the similarities between actinorhizal symbioses and pathogenesis. Nevertheless, enhanced transcription of chitinase genes has been reported for actinorhizal nodules (Kim and An 2002; Fortunato et al. 2007), and Hocher et al. (2006) have shown that transcripts of genes belonging to the functional category of 'defense and cell rescue' occur more often in functional nodules than in roots. Additionally, Tavares et al. (2007) showed that reactive oxygen species (ROS) are produced in nodules. These observations, together with the findings that 1) actinorhizal and legume symbioses had arisen during evolution from a common ancestor (Soltis et al. 1995); 2) their nodule induction pathways share common compounds (Gherbi et al. 2008; Markmann et al. 2008); and 3) at least part of infected cell-specific transcription factors seems to be conserved between the two symbiotic systems (Jacobsen-Lyon et al. 1995; Svistoonoff et al. 2004), suggest that, like in legumes, the symbiosis between actinorhizal plants and *Frankia* might involve mechanisms similar to those found in plant-pathogen interactions.

Although the role of defense-related gene expression during nodulation is not fully understood, several studies have suggested that defense-like events might be involved in the following processes: control of microsymbiont infection, control of nodule number, control of which cells stably accommodate the microsymbiont, protection of nodules against external pathogens, signalling and nodule development (Spaink 1995; Kim and An 2002; Fortunato et al. 2007; Samac and Graham 2007; Tavares et al. 2007).

To provide additional data related to the involvement of defense-related genes during actinorhizal root-nodule symbiosis, and to distinguish between defense-related and developmental control, we have analysed the differential expression of several genes with putative defense-related functions in *Casuarina glauca* nodules induced by *Frankia versus* non-inoculated roots. Four genes, encoding a chitinase (*CgChi1*), a glutathione *S*-transferase (*CgGst*), a hairpin-inducible protein (*CgHin1*) and a peroxidase (*CgPox4*) were found to be up-regulated in nodules compared to roots. In order to complement our data, expression studies were extended to plants subjected to two external stimuli related to the induction of plant defenses, namely salicylic acid, a key molecule in signal transduction pathway of biotic and abiotic stress responses, and wounding. Unfortunately, we were unable to detect the transcripts by *in situ* hybridization of nodule and root sections, which would be of great importance in providing clues about the function these genes. Nevertheless, phylogenetic analysis of the putative proteins encoded by these genes was helpful to find out the function of the closest homolog.

2 Material and methods

2.1 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; http://www.ffp.csiro.au). Plants were grown hydroponically and inoculated with *Frankia* Thr strain as described in Fortunato et al. (2007).

2.2 Wounding and salicylic acid (SA) treatments

For wounding assays, roots and leaves (from non-nodulated plants) and nodules (from plants nodulated by *Frankia*) were mechanically wounded with a needle and harvested 1 h later (Fortunato et al. 2007). For SA treatments, the plant growth medium was supplemented with a 5 mM solution. Roots and leaves (from non-nodulated plants) and nodules (from plants nodulated by *Frankia*) were harvested 6 h later (Fortunato et al. 2007). For both treatments, roots, leaves and nodules from plants growing under normal conditions were sampled and used as negative controls. After harvesting, all samples were immediately frozen in liquid nitrogen and then stored at −80°C until RNA extraction.

2.3 Nucleic acid extraction

C. glauca genomic DNA and total RNA extraction, as well as *Frankia* chromosomal DNA isolation, were performed as described in Fortunato et al. (2007).

2.4 cDNA cloning and sequencing

Total nodule cDNA was synthesized by reverse transcription using total RNA as a template. Specific primers were designed based on the partial cDNA sequences obtained after PCR with degenerate primers (Table 1; *CgChi1*, *CgPr10* and *CgPox1*) or from an EST catalogue (*CgPr4, CgHin1, CgWi, CgGst, CgPox3, CgPox4* and *CgPox5*; Hocher et al. 2006) and PCR was performed using different annealing temperatures (Table 1). Detailed procedures are described in Fortunato et al. (2007). The amplified products were cloned into pGEM-T Easy Vector System I (Promega, Madison, WI), following the instructions of the manufacturer. Six to 10 clones of each PCR product were sequenced.

2.5 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed to analyze the expression patterns of ten defense-related genes (Table 1). cDNA was synthesized from total RNA of roots, leaves and nodules (control, wounded, and SA-treated), as described above. PCR conditions and cycling programs are described in Fortunato et al. (2007). For each gene, the number of reaction cycles is specified in Table 1. Ubiquitin primers (Table 1) 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. Ten µl of the amplified DNA samples were separated on a 1.2% agarose gel. Three independent biological experiments were performed.

2.6 Confirmation of PCR product identity by hybridization

Fifteen µl of PCR product from semi-quantitative PCR were separated on 1.2% agarose gels and transferred to nylon membranes (Roche, Mannheim, Germany) and hybridized with digoxigenin (DIG)-labelled cDNA probes (DIG High prime DNA labelling and detection starter kit II, Roche, Germany) for 16 h at 38°C in a shaking water bath, according to the manufacturer's instructions. After, hybridization membranes were washed as follow: twice for 20′ at 65°C in 2× SSC with 0.1% SDS; twice for 20′ at 65°C in 1× SSC with 0.1% SDS; twice for 20′ at 65°C in 0.5× SSC with 0.1% SDS; 30′ at 65°C in 0.5× SSC with 5% SDS. Detection was performed according to the manufacturer's instructions. Results were photographed, scanned and bands were analaysed using ImageJ (http://rsbweb.nih.gov/ij/). Data were calibrated using ubiquitin expression values. A two-way ANOVA analysis ($P<0.05$) was applied to evaluate expression differences between leaves, roots, and nodules and between treatments, followed by a Tukey test for mean comparison (95% confidence level).

2.7 PCR on genomic DNA

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

2.8 Rapid amplification of cDNA ends (RACE)

RACE-PCR, cloning and sequencing of the PCR products were done according to Fortunato et al. (2007). The gene specific primers used for RACE-PCR are shown in Table 2.

2.9 Sequence and phylogenetic analyses

Sequence reactions were performed at STAB VIDA (Oeiras, Portugal). Sequence similarity search were carried out using BLAST (Altschul et al. 1997) and sequence assembly was performed using CAP3 Sequence Assembly

Table 1 Primer sequences, annealing temperatures (°C) and number of PCR cycles used for cDNA cloning and semi-quantitative RT-PCRs

Gene name	Homology (BLASTx)[a]	Primer sequence	Anneling temperature (°C)	PCR cycles
CgChi1	Chitinase class I	Degenerate:		
		F 5′-TGYTGYWSIVAITWYGG-3′ R 5′-CCIRTIGTYTCRTGISWIGTYTG-3′	40	34
		Specific:		
		F 5′-GGTCGAAGCACGCTCCCGGC-3′ R 5′-GAATAATCCGGACCTGGTGG-3′	50	30
CgGst	Glutathione S-transferase	F 5′-CCAGTACCAGTACAAGGAGGAGG-3′ R 5′-GCTCTCTCTCCAGTGTCTTGAGG-3′	60	35
CgHin1	Hairpin-induced protein	F 5′-TGAGGCCAGGGCATACTACGA-3′ R 5′-AAAAATAAATAAATGGATGAC-3′	45	35
CgWi	Wound inducible protein	F 5′-AGCCCCCGAAATCTAATC-3′ R 5′-TACCCCACACGCATAAACGAG-3′	50	35
CgPox1	Peroxidase	Degenerate:		
		F 5′-TTYCAYGAYTGYTTYGT- 3′ R Oligo $dT_{12–18}$	40	34
		Specific:		
		F 5′-GGGTTGTGATGGTTCAGTGTTGCT-3′ R 5′-CGATGTGAAGAGACCTTGACG-3′	60	30
CgPox3	Peroxidase	F 5′-GCCAGAGAGCGATGTCTCGGC-3′ R 5′-CCTCGAGCTGAATTCCTGTTGG-3′	60	30
CgPox4	Class III plant peroxidase	F 5′-GCACTTCGCCCAATCCATGATC-3′ R 5′-GGTAAACATACAACATGTCC-3′	60	30
CgPox5	Ascorbate peroxidase	F 5′-GGTGAAGAATTACCCATGCGTGAGC-3′ R 5′-CGGTGACTTCAACGGCAACAACC-3′	60	35
CgPr4	Pathogenesis-related protein 4	F 5′-TGGCGTGTGCTTGGTGTTATCTCT-3′ R 5′-AGTTTTAGTCGCCGCAGTCCACAA-3′	60	35
CgPr10	Pathogenesis-related protein 10	Degenerate:		
		F 5′-GARGGIAAYGGIGGICCIGG-3′ R Oligo $dT_{12–18}$	56	34
		Specific:		
		F 5′-GGAATGGGGGGCCGGGAACC-3′ R 5′-AGCATGGCCTTTGGTTATTGGG-3′	60	30
CgUbi	Ubiquitin	F 5′-ATGCAGATYTTTGTGAAGAC-3′ R 5′-ACCACCACGRAGACGGAG-3′		

[a] BLASTx: Translated query *vs* protein databases (Altschul et al. 1997)

Program (Huang and Madan 1999). The following ExPASy (Expert Protein Analysis System) Proteomics Server (http://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), SignalP (prediction of signal peptide cleavage sites) and HMMTOP (prediction of transmembrane helices and topology of proteins).

For phylogenetic analysis, complete amino acid sequences from *Arabidopsis thaliana* were retrieved from Zheng et al. (2004), Tognolli et al. (2002), the supplementary data

Table 2 Primers used for rapid amplification of cDNA ends (RACE)

Gene	5′ RACE	3′ RACE
CgChi1	5′ CCTTCTCGTTCAAACCCTTACCACACTC 3′	5′ ACACATTTGGGGTTGACTACG 3′
CgGst	5′ CTCTCTCTCCAGTGTCTTGAGG 3′	5′ CCAGTACCAGTACAAGGAGGAGG 3′
CgHin1	5′ AAAAATAAATAAATGGATGAC 3′	5′ TGAGGCCAGGGCATACTACGA 3′
CgPox4	5′ GGTAAACATACAACATGTCC 3′	5′ GCACTTCGCCCAATCCATGATC 3′

of Soranzo et al. (2004), www.ncbi.nlm.nih.gov and http://www.arabidopsis.org/-check. Tobacco (*Nicotiana tabacum*) HIN1 cDNA sequence was obtained from NCBI. As outgroups, class tau GST of *Pinus tabuliformis* (AAT69969), an unknown protein from *Picea sitchensis* (ABK21017) with a complete HIN1 superfamily domain and a putative peroxidase from rice (BAD11654) were used in the phylogenetic analyses of CgGST, CgHIN1 and CgPOX4, respectively. These three sequences were also retrieved from NCBI. Sequence analyses were performed as described in Santos et al. (2008) using ClustalX for multiple alignment neighbor-joining analysis.

3 Results and discussion

3.1 Selection of *Casuarina glauca* putative defense-related genes up-regulated in nodules

Based on reports describing the activation of defense-like events during nitrogen-fixing root nodule symbioses and on the sequence information available at the EST catalogue from *Casuarina glauca* (*Institut de Recherche pour le Développement*, Montpellier), we analyzed the expression of 10 putative defense-related genes in *C. glauca* nodules *versus* roots encoding: a wound-inducible protein (*CgWi*), 4 peroxidases (*CgPox1, CgPox3, CgPox4*, *CgPox5*), 2 pathogenesis-related proteins (*CgPr4* and *CgPr10*), a class 1 chitinase (*CgChi1*) and a hairpin-inducible protein (*CgHin1*). Partial cDNA sequences were isolated by RT-PCR. In order to confirm that only one family member of each gene was under analysis, 6 to 10 clones from each PCR product were sequenced and turned out to be identical (data not shown).

The transcriptional activity of 6 genes, *CgWi, CgPox1, CgPox3, CgPox5, CgPr4* and *CgPr10* (Accession numbers CO038263.1, EU346696, CO038208.1, CO038872.1, CO038479.2 and EU346695, respectively), was down-regulated in nodules *versus* roots (Fig. 1). Four genes, *CgChi1* (EU346700), *CgGst* (EU346697), *CgHin1* (EU346699) and *CgPox4* (EU346698), were up-regulated in nodules and selected for further analysis. PCR on *C. glauca* and *Frankia* genomic DNA confirmed that the four selected genes were from plant origin (data not shown).

3.2 *CgChi1*

CgChi1 was represented by a full-length cDNA of 1,631 bp, consisting of a 374 bp 5′-UTR, an open reading frame (ORF) of 960 bp, and a 3′-UTR of 297 bp (data not shown). The putative CgCHI1 protein consisted of 318 amino acids, presenting sequence characteristics of chitin hydrolases (Asensio et al. 2000) and a C-terminal extension motif (GNGLVDTM) similar to that of a tobacco chitinase (Neuhaus et al. 1991) and a class I chitinase from *Elaeagnus umbellata* root nodules (EuNOD-CHT2; Kim and An 2002), which is necessary and sufficient for vacuolar targeting (Neuhaus et al. 1991). In fact, previous phylogenetic studies (Santos et al. 2008) showed that CgCHI1 groups with actinorhizal chitinases, EuNOD-CHT1 and -CHT2 (Kim and An 2002). Although *CgChi1* expression levels were not significantly affected by external stimuli in nodules and roots, transcript levels increased significantly in leaves after SA treatment (Fig. 3). Taken together, these data suggest that *CgChi1* plays a role in nodule defense against external pathogens or in micro-symbiont infection control. In fact, class 1 chitinases are

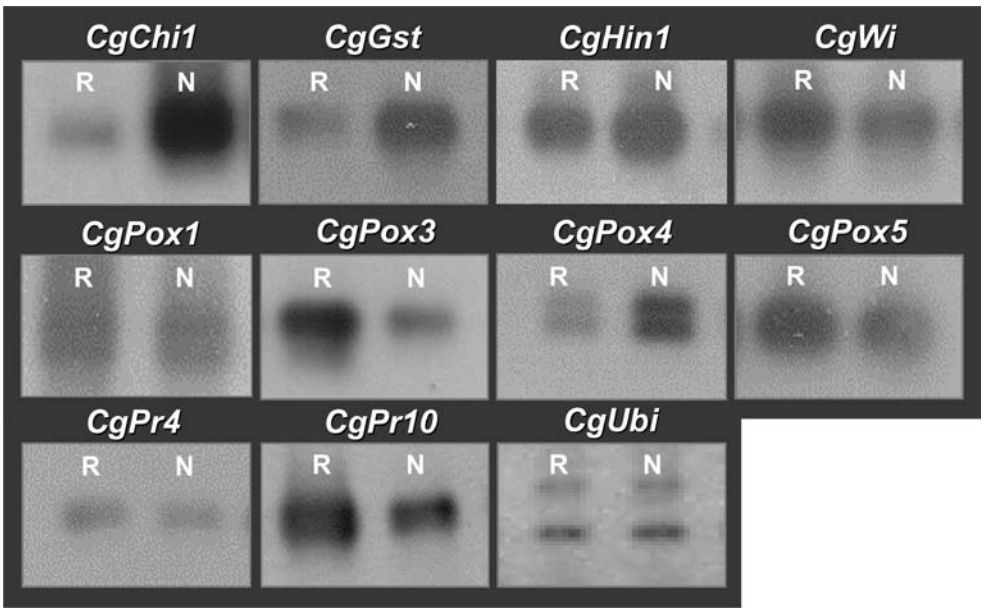

Fig. 1 Selection of *C. glauca* defense-related genes up-regulated in nodules *versus* roots by semi-quantitative RT-PCR, followed by hybridization of PCR products with DIG-labelled probes. *R* roots; *N* nodules

usually associated with defense against pathogenic fungi, namely with the degradation of chitin (Kasprzewska 2003). Additionally, the predicted vacuolar localization of CgCHI1 might be related to the rapid release of large quantities of protein during pathogen infection, as suggested by Neuhaus et al. (1991). Supporting our data are the results from Kim et al. (2005), pointing to the involvement of EuNOD-CHT1 and -CHT2 in defense during root nodule development in *E. umbellata*.

3.3 *CgGst*

CgGst cDNA was 1,027 bp in length, consisting of a 53 bp 5′-UTR, a 314 bp 3′-UTR and a 660 bp ORF encoding a polypeptide of 219 amino acids (data not shown). The encoded CgGST protein has 57–67% amino acid identity with other plant GSTs. It is predicted to be cytosolic and contains the "signature motif" for plant tau GSTs. The phylogenetic profile of CgGST indicates that it groups with ten *Arabidopsis thaliana* class tau GSTs (Fig. 2a), supporting sequence analysis. The expression of *CgGst* was up-regulated in leaves by both wounding and SA treatment, in nodules by wounding, and down-regulated in roots by both treatments, thought significant differences were only observed upon SA treatment in leaves (Fig. 3).

N_2-fixing plants face oxidative risks beyond those associated with photosynthesis. In legume nodules, leghemoglobins, together with high rates of respiration, are believed to be major sources of reactive oxygen species (ROS; Günther et al. 2007); the most common ROS detoxification mechanism in plants is the ascorbate-glutathione pathway, in which GST is a key enzyme (Matamoros et al. 2003). Although the accumulation of ROS in actinorhizal nodules is limited to indirect evidence from one plant species, *Alnus rubra* (Tavares et al. 2007), the fact that similarly to legume nodules, infected cells of *C. glauca* nodules contain large amounts of a class II hemoglobin (Jacobsen-Lyon et al. 1995), makes it likely that in *C. glauca* a similar mechanism of ROS production and detoxification exists and that CgGST is participating in this process. This hypothesis is supported by the up-regulation of *CgGst* in response to SA treatment in leaves, and by the fact that the expression of *AtGSTU19*, encoding a close homolog of CgGST, is supposed to play a role in restricting cellular damage during the resistance response (Wagner et al. 2002).

3.4 *CgHin1*

The *CgHin1* 1,013 bp full-length cDNA sequence consisted of a 83 bp 5′-UTR, a 253 bp 3′-UTR and a 678 bp ORF (data not shown). The ORF encoded a putative polypeptide of 225 amino acid residues with sequence similarity (up to 52%) to several proteins belonging to the large family of NHL which comprises Non-race specific Disease Resistance protein 1 (NDR1) homologs and Harpin-Induced Protein (HIN1) homologs (Zheng et al. 2004; Lee et al. 2006). The proposed topology is similar to that of NDR1 from *Arabidopsis thaliana* (Coppinger et al. 2004): 42 amino acids on the cytosolic side, a transmembrane domain of 22 amino acids and the remaining portion on the apoplastic side. For the phylogeny of CgHIN1, all NHL proteins from *Arabidopsis* and a HIN1 protein from *Nicotiana tabacum* were used (Fig. 2b) CgHIN1 clusters together with NHL10, NHL2, NHL3, NHL9 from *A. thaliana* and NtHIN1 from tobacco. Expression of *CgHin1* was up-regulated in leaves by both wounding and SA treatment and in nodules by wounding (Fig. 3). In SA-treated nodules and wounded roots the expression levels were down-regulated. However, significant effects were observed only in wounded leaves.

NHL genes are normally induced during the hypersensitive response (HR) against pathogen attack, representing strong candidates as elements of signal transduction chains. No information about the involvement of NHL genes in

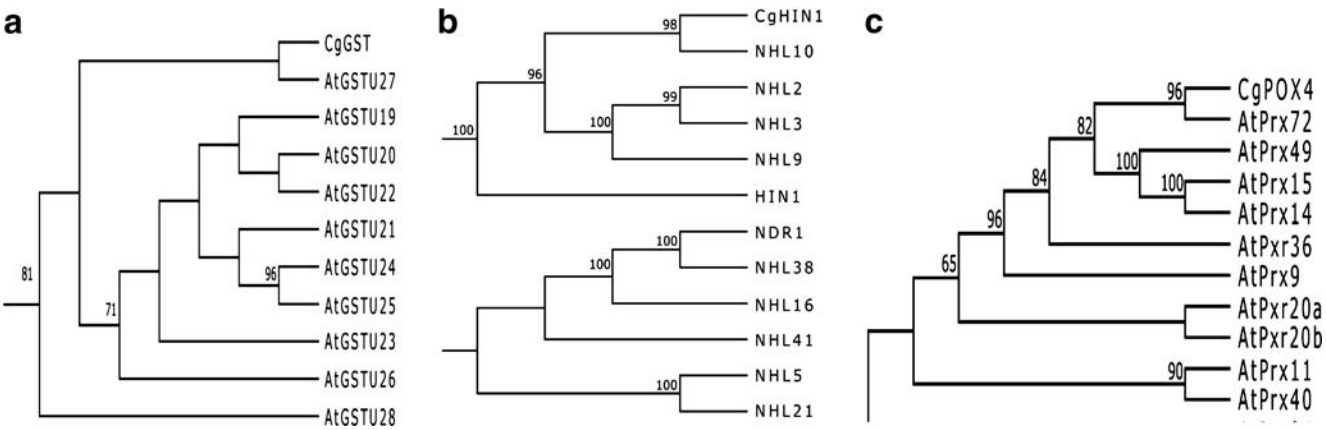

Fig. 2 Partial representation of the phylogenetic tree for the GST family (**a**), NHL family (**b**) and POX family (**c**). Full representations are available at the supplementary data, Fig. S2

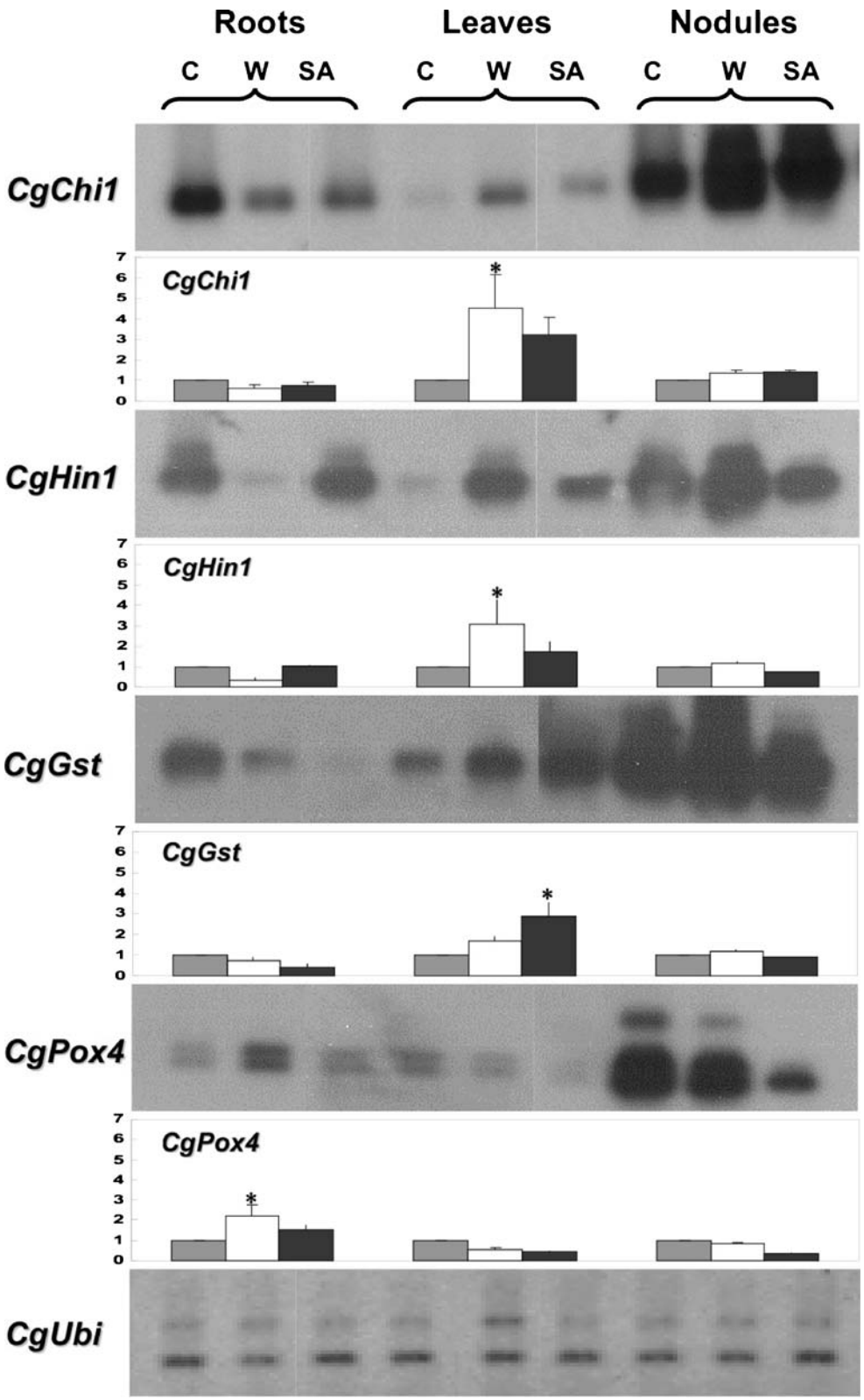

Fig. 3 Expression analysis of *CgChi1, CgGst, CgHin1* and *CgPox4* in roots, leaves and nodules and upon external stimuli (n=3, *error bars* indicate SE; *asterisks* indicate significant differences between treatment and control value with $P<0.05$). *C* control; *W* wounded; *SA* SA-treated

symbiotic processes has been reported. *NHL10*, the closest homolog of CgHIN1, is the ortholog of *NtHIN1* (Zheng et al. 2004), both activated during leaf ageing. The overlap between pathways leading to cell death during both HR and senescence (Quirino et al. 2000) makes it plausible that a single protein might be involved in both functions. By the other hand, *CgHin1* expression in leaves is significantly up-regulated by wounding, and thus presumably by Jasmonic

Acid (JA) signaling, which besides its role in defense signaling has also been implicated in the regulation of senescence (Balbi and Devoto 2008). Although it might be well possible that *CgHin1* is involved in nodule senescence, the similarities between the topologies of CgHIN1 and NDR1, may also suggest that the presence of CgHIN1 is related to pathogen or microsymbiont signaling.

3.5 *CgPox4*

The *CgPox4* full-length cDNA was 1,326 bp in length with a 73 bp 5′-UTR, a 258 bp 3′-UTR and a 995 bp ORF (data not shown). The putative CgPOX4 polypeptide had 332 amino acid residues, with significant sequence identity (up to 76%) to a number of class III plant peroxidases. In common with other class III plant peroxidases, CgPOX4 had a signal peptide for uptake in the endoplasmic reticulum and a predicted extracellular localization. According to the phylogenetic analysis (Fig. 2c), CgPOX4 forms a small cluster together with AtPrx15, AtPrx14, AtPrx49, AtPrx36, AtPrx72, AtPrx9, AtPrx20 and AtPrx10 (Tognolli et al. 2002). Expression of *CgPox4* was down-regulated in leaves and nodules by both wounding and SA treatment, and up-regulated in roots by both treatments (Fig. 3). However, only in wounded roots were the expression levels of *CgPox4* significantly higher than those of control healthy roots.

Peroxidases have been implicated in a broad range of physiological processes such as auxin metabolism, cross-linking of cell wall components or in defense against pathogens (reviewed by Passardi et al. 2005). *CgPox4* expression in nodules was down-regulated by SA and wounding. By the other hand, the pair CgPOX4/AtPrx72 is closest to AtPrx49. The function of *AtPrx72* is unknown but *AtPrx49* is induced during the interaction with root knot nematodes and involved in lignification (Vercauteren et al. 2001). Thus, it might be well possible that CgPOX4 is involved in the cell wall modifications that take place during nodule development to accommodate symbiotic *Frankia* bacteria.

4 Conclusions

Two opposing, yet parallel processes take place during the course of nodulation: the induction and suppression of plant defenses. Suppression of plant defenses is essential for the establishment of effective symbioses (Mithofer 2002) while induction of defense mechanisms has been associated with infection control and defense against external pathogens (Samac and Graham 2007). With this study we intended to evaluate the possible roles of putative defense-related genes in actinorhizal nodule development and/or defense. The expression of four genes, *CgChi1, CgGst, CgHin1* and *CgPox4* was up-regulated in mature nodules compared to roots suggesting a specific function in the symbiotic process.

None of the four genes examined showed significant changes in expression levels in response to wounding or SA treatment in nodules. However, it should be noted that the basal expression levels of *CgChi1, CgGst* and *CgHin1* in nodules were higher than their induced expression levels in leaves, respectively. Similarly, the basal expression level of *CgPox4* in nodules was much higher than its induced expression level in wounded roots. It is known that genes encoding proteins involved in defense are not necessarily required for defense processes in all organs. For example, *pdf1.2* is induced by JA in leaves but not in roots (Badri et al. 2008). Similarly, Arabidopsis *NDR1* is induced in response to *Hyaloperonospora parasitica* in leaves, but not in roots (Hermanns et al. 2003). Since none of the four genes under investigation was significantly induced by SA treatment or wounding in nodules, it might be concluded that they do not play a role in defense during symbiosis. However, their elevated expression levels in nodules compared to roots and leaves can be interpreted to indicate that defense is constitutively up-regulated in nodules. Combining the expression data with the phylogenetic analysis and the available information on gene function for other plant species, we propose that *CgChi1* and *CgGst* are involved in defense or infection control, *CgPox4* participates in nodule development and for *CgHin1* the question "defense, development or both" remains open. Gene regulation studies with promoter-GUS fusions in transgenic plants as well as the biochemical characterization of the encoded proteins are in progress to further clarify the role of these genes. Additionally, transcriptomic and proteomic approaches are being implemented in our labs in order to extend this work and to get a whole and integrated picture of the involvement of putative defense genes during nodulation.

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