SYMBIOSIS (2006) 42, 25–37 ISSN 0334-5114

Cloning of four chitinase genes and a lectin gene in *Galega orientalis*

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(Received March 21, 2004; Accepted July 15, 2006)

Abstract
The legume goat's rue (*Galega orientalis*) and its microsymbiont *Rhizobium galegae* have an unusually strict host-bacteria specificity. The reason for this is not known. We have identified and analysed six genes in *G. orientalis* assumed to be involved in the recognition processes between legumes and rhizobia. The genes were isolated from a library made from nodulating roots. The genes were identified by sequence analysis as class Ia, Ib and class IV chitinases, a lectin, and a presumptive defensin. The chitinase and lectin sequences were used to complete phylogenetic trees. In the phylogenetic tree, the *G. orientalis* lectin gene is placed in the same group as *Pisum sativum Nlec*, *Blec* and *Robinia pseudoacacia LEC3*. These genes have been suggested to be involved in different developmental processes. Defensins are involved in several defence functions, and recently there have been a few publications on defensins involved in nodulation. 2 The activity of chitinases in different tissues and in nodulated roots was investigated by western blots and activity gels. *G. orientalis* has at least four different active chitinases. The sequences described in the article have the following GenBank accession codes: AY253984, AY253985, AY253986, AY253987, AY253988, AY333428

Keywords Chitinase, defensin, *Galega orientalis*, legume lectin, nodulation, *Rhizobium* and symbiosis

1. Introduction

The interaction and communication between a legume and its corresponding *Rhizobium* microsymbiont will eventually result in the formation of the nitrogen-fixing plant organ, the nodule. During the nodule organogenesis, there are several steps controlling which combinations of legume and rhizobia will result in a fully functioning nodule, capable of efficient nitrogen fixation. The host-microsymbiont specificity varies widely, and several examples of strict host-range are long known within the legumes as well as the rhizobia (Wilson, 1939; Perret et al., 2000). On the other hand, the genus *Galega* has been described by some authors to have an unusually narrow host-range (Perret et al., 2000), making it especially interesting as material for studying what determines host-range. Goat's rue (*Galega orientalis* Lam.) is a perennial leguminous plant, originating from Caucasus. The microsymbiont of *G. orientalis* is *Rhizobium galegae* bv. (biovar) *orientalis*. The only other legume this rhizobium nodulates is another member of the Galegeae tribe, *G. officinalis*, although the nodules formed are not effective in nitrogen fixation. Similarly, *R. galegae* bv. *officinalis* induces effective nodules only on *G. officinalis* (Radeva et al., 2001). The reason for this unusually strict host specificity is not known and is presumed to involve different factors.

We have identified and analysed different plant genes in *Galega* that may be involved in the development of nodules or in the recognition process. The identified genes are chitinases, lectins, and defensins. None have been previously described in *Galega*. Chitinases belong to the group of Pathogenesis Related proteins (PR-proteins) expressed in plants after elicitation by various factors or in some cases developmentally regulated. Chemically and structurally, the chitinases are divided into 5 different classes, class I to V. Classes I, II and IV share homologies, while classes III and V have no sequence or structural similarity with the others. Class I chitinases have a chitin binding domain, a variable hinge and a catalytic domain.

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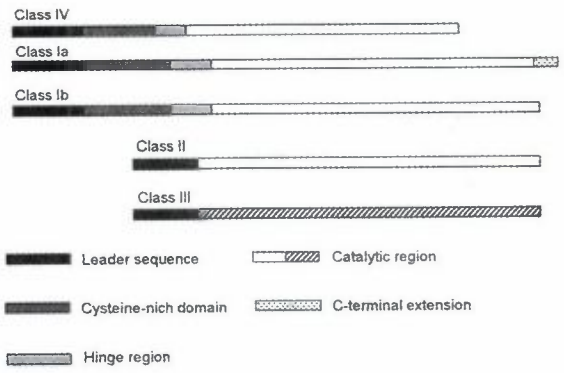

Figure 1. A representative of the different classes of chitinases, where the different domains and signal peptides can be seen. The difference between class 1a and 1b (in this article referred to as I*) is the C-terminal signal peptide directing the protein to the vacuole (modified after Collinge et al., 1993).

Most chitinases also have a C-terminal extension directing them to the vacuole as illustrated in Fig. 1. According to Neuhaus et al. (1991) the C-terminal extension is sufficient as well as necessary to target the protein to the vacuole. While class II chitinases lack the chitin-binding domain and the C-terminal extension present in class I, class IV chitinases are very similar to class I chitinases, but with 4 deletions (Ancillo et al., 1999; Davis et al., 1991; Suarez et al., 2001).

The expression of the class I chitinases increases after fungal infections and increases transiently in the early nodulation process. In arbuscular mycorrhizal infections of *Medicago truncatula,* classes I, II and IV chitinases remain at a low basal level or are suppressed by the fungi, while class III chitinases increase (Salzer et al., 2000). In *Sesbania rostrata*, a class III chitinase has been found to increase after nodulation (Goormachtig et al., 1998). Class IV chitinases increase early in nodulation and continue to increase. The class IV chitinases are often expressed in the endodermis surrounding the infected tissue as well as in the rest of the root, suggesting a protective function against fungal infections. Publications on expression of Class V chitinases during nodulation are limited, but class V was recently connected with nodulation in *M. truncatula* (Salzer et al., 2004). We have isolated four chitinase cDNA-clones in total, and also shown a tissue-specific expression of chitinases of *Galega*.

The elicitor of the chitinases during the initial stage of nodulation is likely to be bacterial Nod (nodulation) factors (Ovtsyna et al., 2005; Salzer et al., 2004). The Nod factors are lipo-chito oligosaccharides, consisting of a chitin backbone carrying various species-specific decorations and substitutions. Thus, the bacterial Nod factors are substrates for this type of PR-protein, as shown by several authors (e.g., Ovtsyna et al., 2000). The chemical structure of the Nod factors influences the degree of their degradation by a specific chitinase. Hence, the bacterial Nod factors and plant chitinases are assumed to be involved in the specificity of the interactions between legumes and rhizobia (Perret et al., 2000; Staehelin et al., 1994). The Nod factors of the rhizobia of the galegoid group (the clade including Galegeae, Trifolieae and Vicieae) are rather unusual compared to other rhizobiaceae, as they are all alpha-unsaturated. Nod factors of *R. galegae* strain H1207 contain an O-acetyl group on a non-terminal chitin backbone residue, which is a unique character for Nod factors, possibly giving them a unique resistance to chitinases from *G. orientalis* (Yang et al., 1999). Chitinases from other legumes have been isolated, but no chitinases from *G. orientalis* have so far been described.

Legume lectins are one of the most common proteins in legume seeds (Sanz-Aparicio et al., 1997). Lectins are chemically characterised as proteins with at least one binding site that binds carbohydrates or polysaccharides reversibly and non-catalytically (Hirsch, 1999; Naeem et al., 2001). Legume lectins are divided into seed lectins and vegetative lectins, according to the expression pattern (Esteban et al., 2002).

There have been several publications on the involvement of lectins in the recognition and binding processes between the legume and the microsymbiont during nodulation (Hirsch, 1999; Kardailsky et al., 1996). In *Medicago truncatula*, two lectins, *Mtlec1* and *Mtlec2,* 5 were shown to be transcribed in dividing cells of the nodule primordium (Bauchrowitz et al., 1992). The two isoforms of the pea seed lectin (PSL) are expressed in roots and secreted into the rhizosphere, where it may function to accumulate *Rhizobium* cells. The two vegetative pea lectins *Blec* and *Nlec* lack an important glycine (in our alignment; Gly-246) present in PSL, presumed to be involved in sugar binding (Kardailsky et al., 1996). Transforming alfalfa with the PSL gene enhanced the colonization of the transgenic alfalfa by the pea-specific *Rhizobium* (vanRhijn et al., 2001). Similar approaches have also successfully been used in other legumes (e.g. Diaz et al., 1986, 1989, 2000; vanEijsden et al., 1995; vanRhijn et al., 1998).

Defensins exist in mammals, insects and plants and are involved in the initial defence reactions with activity against bacteria and fungi. They are small, basic, cysteine-rich peptides, structurally similar and with conserved regions. In plant defensins, eight cysteines form four disulfide bonds. They are mainly expressed in plants during stress, ripening of fruits and in reproductive tissue (Raj and Dentino, 2002). Some defensins have homology to plant proteinase inhibitors and other antimicrobial peptides. The plant defensins in most publications have been analysed for their antimicrobial activities, and their involvement in pathogenic defence is well established (Thomma and Cammue, 2002). There have, however, been a few reports of defensins specifically involved in nodulation. Two nodulin genes (*MtN1, MtN13)* with homology to plant defensins and other defence proteins have been found in *M. truncatula* by differential screening (Gamas et al., 1998;

Werner et al., 2002). Recently several different small cysteine-rich peptides expressed in different developmental stages of root nodules have been found in *M. truncatula, G. orientalis* (Graham et al., 2004; Mergaert et al., 2003) and *P. sativum* (Gamas et al., 1998). These peptides have six or sometimes eight cysteines, occasionally also lacking one or more of the conserved cysteines.

2. Materials and Methods

Library

A ZAPII phage library (Kaijalainen et al., 2002) was used for the screening. The cDNA had been isolated from nodulated *G. orientalis* roots, inoculated with *R. galegae* bv. *orientalis* (ATCC43677, HAMBI540) three days after germination. Roots were harvested twice: two days after inoculation and 21 days after nodulation. The plants used for inoculation were grown and watered with Jensen broth without nitrogen according to Kaijalainen et al. (2002).

Probes

Probes for class I chitinase were obtained from cDNA clones from *M. sativa* (U83592) and *P. sativum* (X63899) by courtesy of S. Purevin, C. Vance and D. Collinge, (Vad et al., 1991, 1993; D. Collinge's cDNA clone collection: www.plbio.kvl.dk-dacoj3/accnos.htm). The inserts of the cDNA clones were cleaved out, separated on an agarose gel and purified using a QIAEXII Agarose Gel Extraction Kit. The fragments were labelled using Prime-It®II Random Primer Labelling Kit from Stratagene. The probes were purified by ProbeQuant™ G-50 Micro Columns (Amersham Biosciences, Uppsala, Sweden).

The probe used to screen for class IV chitinases was made by using degenerate PCR primers against a plasmid library from *G. orientalis*. The degenerate primers were made according to Salzer et al. (2000):
5' CTITGYTGYWSIMRITWYGGITWYTGYGG 3' and
5' TGTCCIGTATCRTGISWIRHRTGIGCRAA 3'
where R=A/G, Y=C/T, H=not G, W=A/T, M=A/C, S=G/C, I=inosine. In the original article, these primers were used with *M. truncatula* cDNA and resulted in a 310 bp PCR fragment.

The plasmid library was constructed by mass excision from the phage library according to the Stratagene (www.stratagene.com) protocol. To the agar plates with the excised bacterial colonies, LB was added and the plates were put on a rotary shaker for 3 h at 4°C. Solutions from the plates were combined and incubated at 37°C on a rotary shaker for 2 h. Plasmids were prepared using Jet Quick Maxi Prep kit from Genomed. The total plasmid library contained about 358,000 clones. The PCR protocol was optimised as follows: 94°C 6 min, then 35 cycles of 94°C 45 s, 51°C 30 s, 72°C 40 s followed by 10 min at 72°C. The PCR mix contained 0.2 mM dNTP mix (Invitrogen), 1.5 mM $MgCl_2$, 5 µM primer 1, 1 µM primer 2, 0.625 U Taq polymerase (Invitrogen), and 13.6 ng/µl of the plasmid library. The PCRs were run in a PC-960G Gradient thermal cycler (Corbett Research, Mortlake, Australia).

Screening of library

250,000 plaques were screened according to the ZAPII protocol from Stratagene (www.stratagene.com). XLI Blue MRF' was used as host strain. From each plate, nylon filters were lifted according to the Hybond™NX-protocol from Amersham Biosciences and then UV-crosslinked. The filters were prehybridised in a hybridisation solution containing Denhardt's solution (Sambrook et al., 1989), for 2 h before the probe was added. The alfalfa probe (described above under "Probes") was incubated at 65°C overnight, the pea chitinase probe at 60°C and the class IV probe at 55°C.

The membranes were washed twice in 2xSSC, 0.5% SDS for 15 min and then for 10 min at 60°C, then for 10 min in 0.2xSSC, 0.1% SDS at 60°C, and for 15 min in (0.1xSSC 0.1% SDS) at 55°C. Hyperfilm MP (Amersham Biosciences) was left on the membranes 8 at –80°C overnight. The films were developed and aligned with the filters. Plaques were picked and used for double or triple screenings, which eventually allowed single positive plaques to be isolated. Single excisions were made according to the Lambda ZAP II Library protocol (www.stratagene.com). Colonies were picked and used for minipreparations (Sambrook et al., 1989).

Sequencing

T3, T7 and M13-reverse primers (Stratagene) were used to sequence the clones. Sequencing was done using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA), and the capillary electrophoresis was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) at the BM-lab, Lund. Two µl Big Dye3 reaction, 6 µl sequencing buffer (200 mM Tris-HCl, 5 mM $MgCl_2$, pH 9.0) 3.2 pmol primer and 450 ng of the plasmid preparation were used in each reaction. The PCR-product was sequenced in a similar way. 12.4 pmol of primer 1 and 4.8 ng of the PCR-product were used.

These sequences were then used to search the BLAST database (Altschul et al., 1997, http://www.ncbi.nlm.gov/BLAST/).

Phylogenetic analysis

Amino acid sequences were first aligned using ClustalW (Thompson et al., 1994) and trees were constructed by

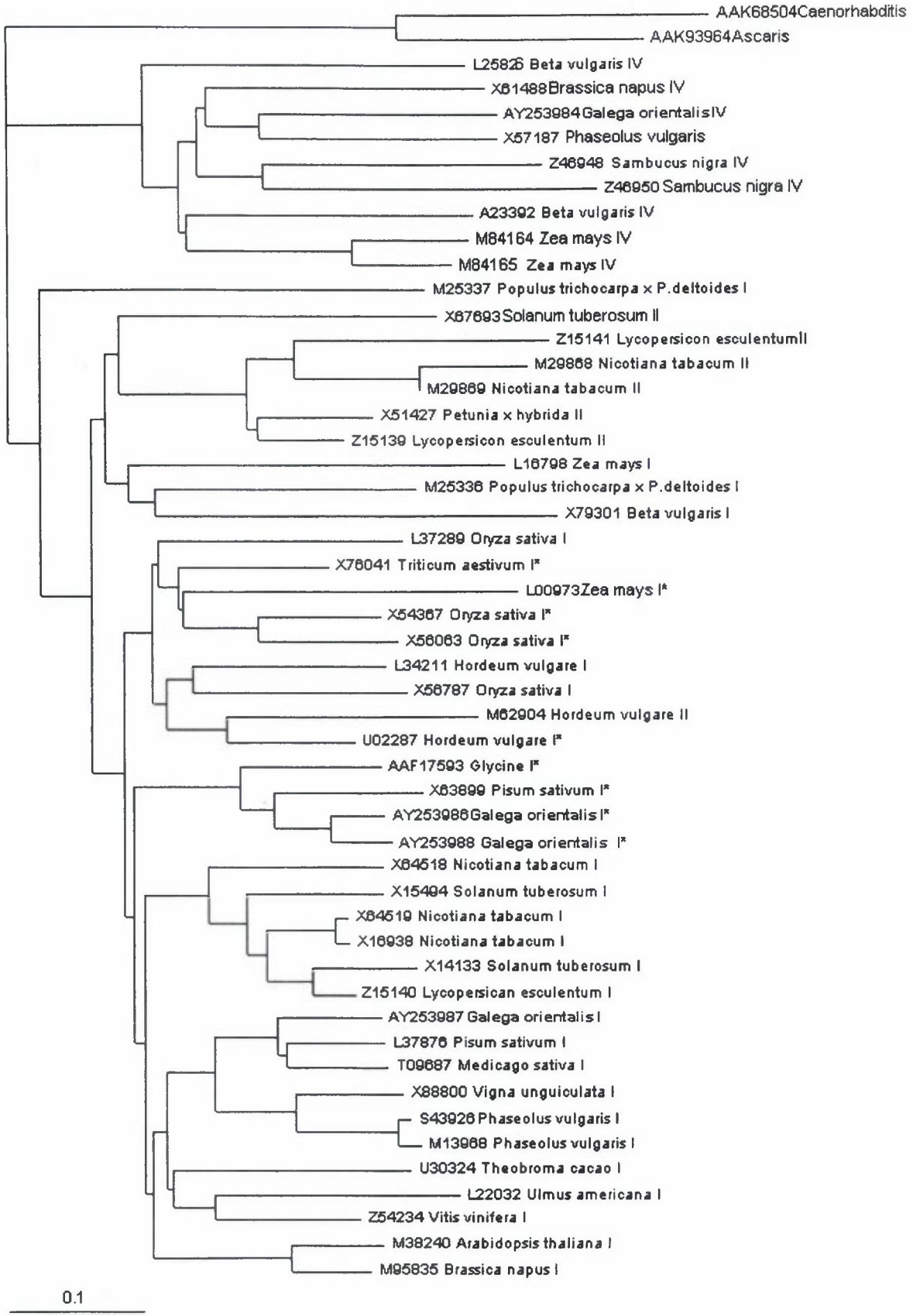

Figure 2. Phylogenetic tree based on a tree made by Hamel et al. (1997) but including the four new chitinases from *G. orientalis*, a chitinase from *M. sativa* and two nematode chitinase sequences. The scale indicates 0.1 amino acid divergence in 100 amino acids.

maximum parsimony, Neighbour-joining and maximum likelihood methods using the PAUP software (version 4.0b1, from D.L. Swofford, Smithsonian Institution, Washington, DC). The entire protein sequences were used for the alignment. The degree of statistical support for branches in the phylogeny was determined (Felsenstein, 1985) by analyzing 1,000 bootstrap replicates of the data. For 9 cross reference, Neighbour-joining trees were constructed using the Phylip program in the GCG package and also the interactive site of ClustalW at (http://www.ebi.ac.uk/clustalw/index.html).

The alignment is not shown due to its large size. The tree shown in Fig. 2 is a Neighbourjoining tree. The tree files were viewed in Treeview 1.6.6 and further edited in MacDraw. The chitinase tree was based on a tree made by Hamel et al. (1997). The four chitinases from *G. orientalis* and a sequence from *M. sativa* were added. Two nematode sequences with homology to chitinases from plants (AAK68504, *Caenorhabditis elegans* genomic sequence and AAK93964, *Ascaris suum* putative chitinase) were used as outgroup. The phylogenetic tree with the lectin sequences was made as described for the chitinase sequences. The tree was based on a tree previously published (Kardailsky et al., 1996). The defensin alignment was also done as described above.

Plant material

Plants for western blots, IEF-gels and RT-PCR were grown in a growth chamber under a 12 h light/12 h dark period at 20±2°C. Normal daylight was supplemented with helium lamps resulting in an average photosynthetic flux density of 150 μs^{-1} m^{-2}. The seeds were sterilised in 95% ethanol for five minutes and commercial bleach for 15 minutes and then washed five times in sterilised water. The seeds were planted in a soil-sand mixture. Plants used to isolate very young nodules and roots were grown in vermiculite. The rhizobium culture was grown in TY medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 28°C until an OD600 of 1.0. The culture was pelleted and rinsed with sterile distilled water and resuspended in distilled water to give an OD of 0.1–0.2. The culture was added directly to the vermiculite either when seeds were planted (for western blots) or when the plants were 10 days (for RT-PCR).

Isolation of PR-proteins

Six g of root, leaf or flower tissue were homogenised in liquid nitrogen. The material was further homogenised for two min in a solution containing 84 mM citric acid, 32 mM Na_2HPO_4 and 14 mM 2-mercaptoethanol (pH 2.8). After centrifugation (18,000 g), the supernatant was filtered through Whatman IF filter paper. The protein solution was dialyzed in a Spectrapor 3500 tube against 1 mM Tris, pH 6.8 at +4°C overnight. The preparation was concentrated by placing the dialysis tubes in PEG (MW 20,000) until the volume was reduced to 0.5–1 ml.

Expression analysis

Proteins from nodulated and non-nodulated roots were prepared from 20 days old plants. A SDS PAGE (polyacrylamide gel electrophoresis) (30%) was run (Laemmli, 1970) using 1 µg protein in each lane. The protein bands were electrotransferred to a Hybond™ ECL™ nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). The blots were incubated with a polyclonal antibody against a barley class II chitinase (Liljeroth et al., 2001). The Chemoluminescence ECL+Plus Western Blotting Kit from Amersham Biosciences was used to visualize the bands.

A 30% isoelectric focusing gel (IEF) containing Pharmalyte 8-10.5 (Amersham Biosciences, Uppsala, Sweden) with 3 µg protein (determined according to Bradford, 1976) was run and an overlay gel containing glycol chitin was pressed against the native IEF-gel during incubation at 37°C. After incubation, the overlay gel was stained with calcofluor white (Sigma) (0.1 mg/ml in Tris, pH 8, 0.5 M) and placed on a UV-table to visualize the bands (Pan et al., 1991). The roots used for the protein preparation for the chitinase assay came from a several years old plant with large red branched nodules.

Expression analysis of mRNA by RT-PCR

Roots were harvested and freezed at –80°C at 5, 10, 20 or 30 days after the day of inoculation. Roots from non-nodulated plants were harvested at the same timepoints. From now on the plants will be named according to their ages after infection of *Rhizobium galegae*. RNA from roots with and without nodules was isolated with Trizol reagent (Invitrogen) according to the manufacturer. RNA products were separated on a 1% agarose gel and visualized by staining with ethidium bromide. RNA ladder (0.24–9.5 kb) was used as the molecular weight standard.

Table 1. The lengths of the base pair sequences of the genes investigated by RT-PCR. Names of primers used for amplification are indicated in parenthesis after the length.

Gene	Length in base pairs
Chitinase class I	194 (3s–3s1)
Chitinase class IV	Approx. 750 (4s–4s1)
Lectin	214 (3L–Lec1)
Defensin	399 (with primer Da–Db)
	Approx. 180 (with primer def1–def2)

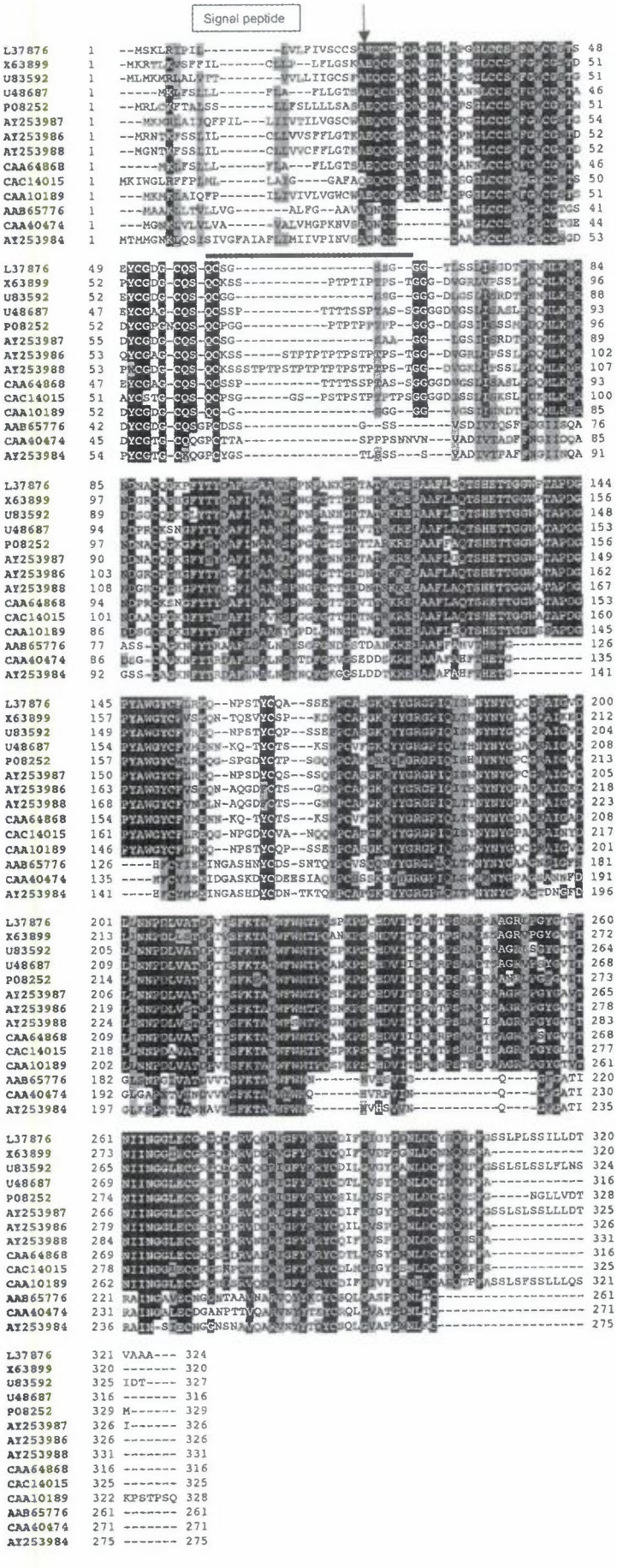

Figure 3. Clone AY253984 (*Go-chtIV*), AY253986 (*Go-cht1b*), AY253987 (*Go-cht1a*), AY253988 (*Go-cht1b2*) are the isolated chitinases from *G. orientalis*. The hypervariable hinge is marked with a solid line. Alignment was made with ClustalW in BioEdit v5.0.9. The first variable part is the signal peptide. L37876 *Pisum sativum*, X63899 *Pisum sativum*, U83592 *Medicago sativa*, U48687 *Castanea sativa*, *P08252 Nicotiana tabacum*, CAA64868 *Castanea sativa*, CAC14015 *Vitis vinifera*, CAA10189 *Cicer arietinum*, AAB65776 *Vitis vinifera* IV, CAA40474 *Phaseolus vulgaris* IV.

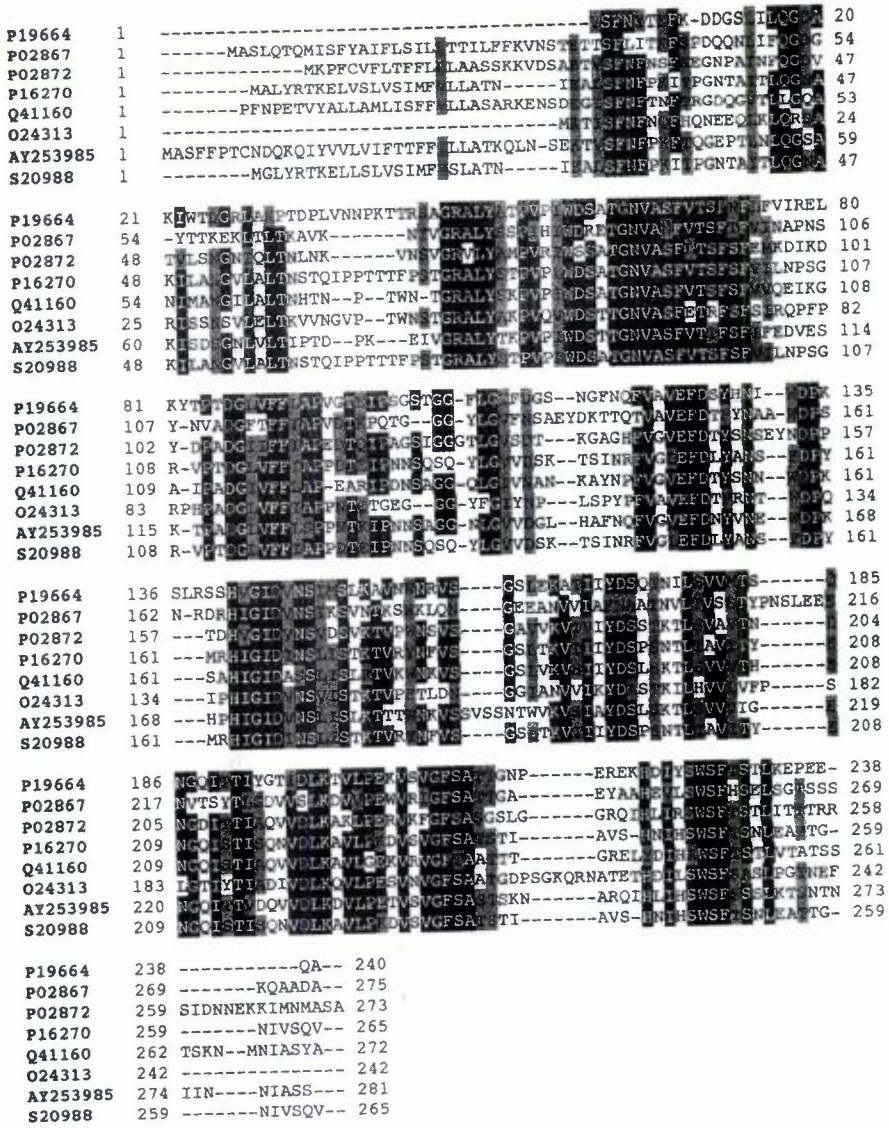

Figure 4. The *G. orientalis* clone *Golec* (AY253985) aligned with other lectin proteins. P19664 *Lotus tetragonolobus*, P02867 PSL from *Pisum sativum*, P02872 *Arachis hypogaea*, P16270 *Nlec* from *Pisum sativum*, Q41160 Putative bark agglutinin LECRPA3, *Robinia pseudoacacia*, O24313 *Psophocarpus tetragonolobus*, S20988 *Blec* from *Pisum sativum*.

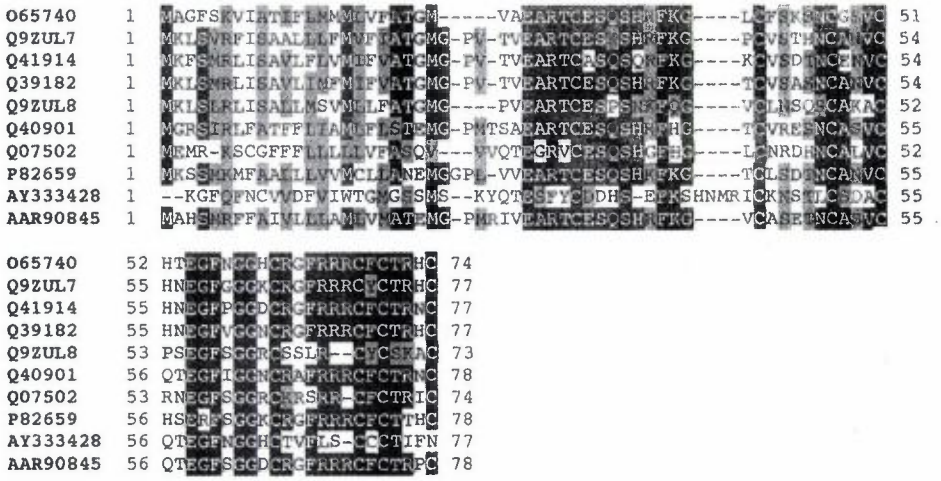

Figure 5. n93 (AY333428) aligned with proteins from the defensin family. O65740 defensin J1-2 from *Capsicum annuum*. Q9ZUL7 thg3 from *A. thaliana*. Q41914 thg2 from *A. thaliana*. Q39182 thg1 from *A. thaliana*. Q9ZUL8 thg4 from *A. thaliana*. Q40901 defensin from *Petunia integrifolia*. Q97502 a sulfur-rich protein/proteinase inhibitor P322 from *Glycine max*. P82659 flower-specific defensin SD2 from *Helianthus annuus*. AAR90845 defensin from *Capsicum annuum*. AY333428 our putative defensin from *G. orientalis*. The L, M and V-rich first part of the alignment contains the signal peptides.

DNA amplification

According to SuperScript™ One-Step RT-PCR with Platinum® Taq (Invitrogen) cDNA was synthesized from mRNA and immediately followed by PCR amplification. The program was as follows: 48°C for 30 min, 94°C for 4 min, 35 cycles with 94°C for 30 s, 55°C for 40 s, 72°C for 1 min. The program ended with 72°C for 5 min and 25°C until the tubes were removed from the machine. To ensure that the PCR reactions were specific for RNA, reactions using Taq polymerase instead of SuperScript polymerase were made. Reactions without template were made to exclude contamination.

The primers and antisense primers used in the different samples were: 3L and lec1 (lectin gene), 3s and 3s1 (chitinase gene class I), Da and Db (defensin gene), 4s and 4s1 (chitinase class IV gene), and control1 and control2 (ubiquitin gene, primer sequences not shown) (see Table 1). After separation of the PCR products on an 1% agarose gel electrophoresis they were visualized by staining with ethidium bromide. DNA molecular Weight Marker X was used as the molecular weight standard.

3. Results

Screening

Class I chitinases: Out of the 250,000 plaques, 50 positive plaques were selected. Of these, 29 plaques were used for second and/or third screenings. Eventually 15 clones were partially or fully sequenced, of which ten were concluded to be chitinases. When analyzed in BLAST, three different chitinases could be identified: *Go-cht1a* (AY253987), *Go-cht1b* (AY253986) and *Go-cht1b2* (AY253988) (Fig. 3). The *Go-cht1b* and *Go-cht1b2* lack the C-terminal vacuolar signal, and are therefore probably class Ib chitinases. In *Go-cht1a*, a vacuolar signal is present (the last 13 amino acids). The chitinase clones *Go-cht1b* and *Go-cht1b2* are very similar and differ from each other at 24 amino acid positions in the chitin-binding domain and catalytic domains. The hinge region is also longer in *Gocht1b2* (AY253988; amino acid 64–87). A vacuole signal can be seen in the end of the proteins, and there is also a signal peptide in the beginning of the proteins.

Class IV chitinases: Out of the 250,000 plaques screened, 12 were used for further screening and sequencing. Two of the clones coded for a chitinase (see Fig. 3, *Go-chtIV*, AY253984). Based on the high homology to class IV chitinases, we suggest that it is a class IV chitinase. Class IV chitinases are very similar to class I chitinases, but have 4 deletions, also present in our clone. Our clone has no vacuolar signal, corresponding with the fact that most class IV chitinases are extracellular (Lange et al., 1996). The four deletions can be seen after amino acids 36, 141, 220 and 277 in *Go-chtIV* (AY253984).

Galega lectin gene

In the screening process, two other genes were also found. One of them has high homology to legume lectins. It contains the legume lectin *a*-domain (amino acids 233–271) and legume lectin *b*-domain (amino acids 45–224) and is characterised as a legume 13 lectin by structure databases such as SMART. When compared to NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/BLAST/), the *Galega* domains show a very high degree of homology to the consensus sequences of legume lectins (about 60% at the amino acid level to the most similar legume lectins). When used in BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) the sequences with highest homology to our sequences are LECRPA3 from *Robinia pseudoacacia*, a vegetative lectin from *Cicer arietinum* (chick-pea), a galactose-binding lectin from peanut, and a lectin from *Lotus japonicus* (Fig. 4). Therefore we conclude that the gene is a legume lectin gene (AY253985 *Go-lec*).

Other genes

One short sequence was also isolated from the root library, n93 (Fig. 5, AY333428), which when translated and used in BLAST-searches, showed a weak homology to plant defensins (earlier called g-thionins). When analyzed in different protein databases, it was classified (with a low score) as belonging to the arthropod type of defensins.
(Pfam:http://www.sanger.ac.uk/Software/Pfam/index.shtml,
Prodom:http://prodes.toulouse.inra.fr/Prodom/2001.1/html/home.php,
TIGR: http://www.tigr.org/TIGRFAMS/index.shtml).

In the SMART database, it was classified (also with a low score) as a member of the Knot1-family of proteins (http://smart.embl-heidelberg.de/). The similarity between all the members and the consensus sequence is very low, except for certain amino acids, such e.g., cysteines, which are conserved.

We have aligned (Fig. 5) the n93 sequence with four different defensins from *Arabidopsis* (Thomma and Broekaert, 1998), two from bell pepper (*Capsicum annuum*), one from *Petunia integrifolia*, a sulphur-rich protein from soybean (*Glycine max*) and one from 14 *Helianthus annuus*. *A. thaliana* has five different defensins that are differentially expressed in different tissues (Thomma and Broekaert, 1998).

Phylogenetic trees

The phylogenetic tree made for the chitinases is presented in Fig. 2. Three out of the four identified

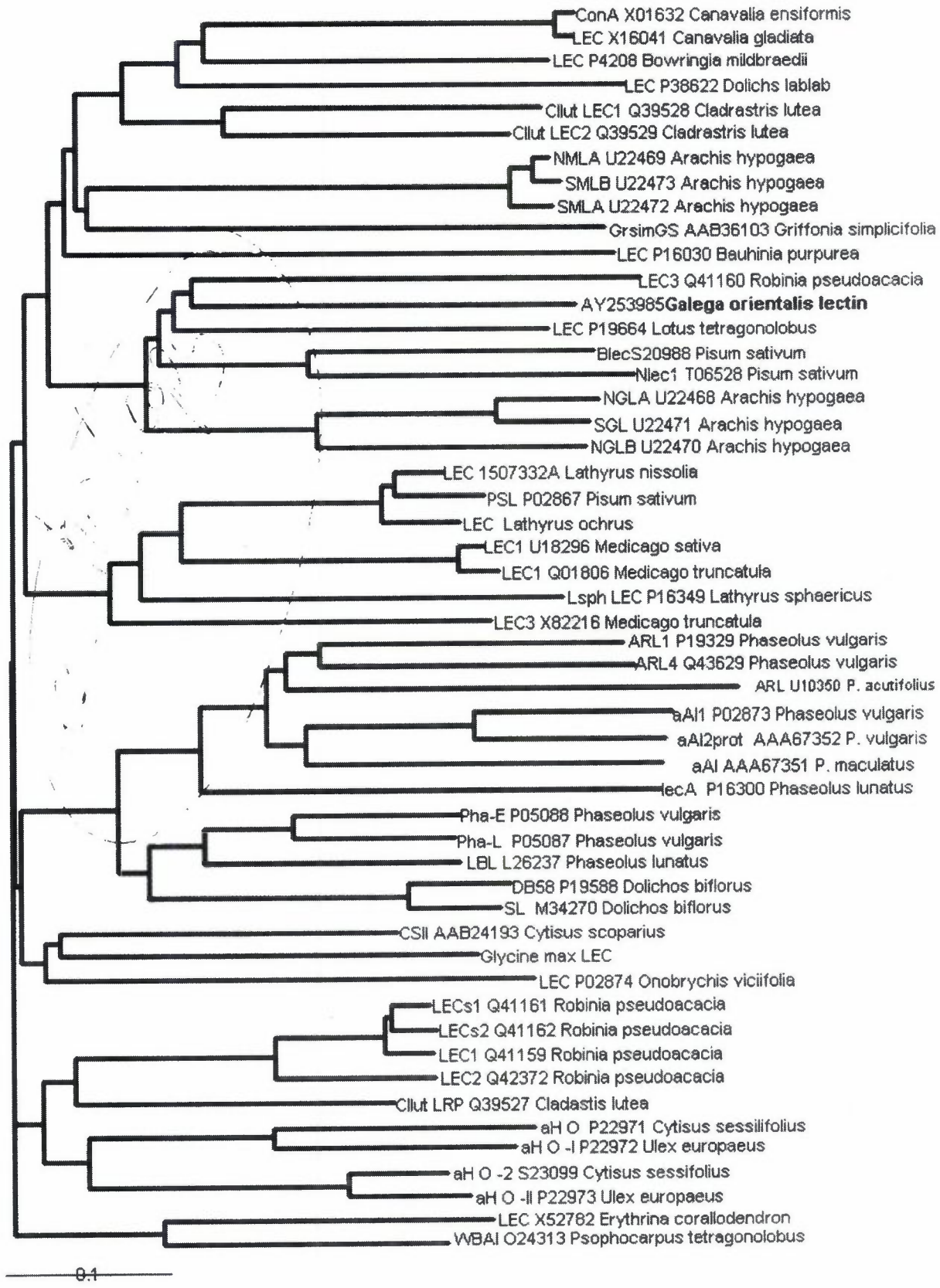

Figure 6. Phylogenetic tree of legume lectins. The tree includes five sequences from *Robinia pseudoacacia,* of which four of them are placed together in one branch, one of them (LecRPA3) is placed together with the lectin from *Galega orientalis* (AY253985) and *PsNlec1*. The lectins in this group are thought to have a role in development (Esteban 2002). The scale indicates 10 amino acids divergence in 100 amino acids.

chitinase clones from *G. orientalis* could be classified as belonging to class I chitinases, whereas one was a class IV chitinase based on comparisons to previously published sequences. Previous phylogenetic relationships of chitinases were confirmed by the addition of the *Galega* sequences (Hamel et al., 1997). The class I and IV chitinases are placed together with chitinases from the respective classes. *G. orientalis* class Ia and Ib (I* in the phylogenetic tree, Fig. 2) chitinases are placed in the same clade as the *P. sativum* sequences. The asterisks indicate class I chitinases without the C-terminal extension. The trees looked very similar using different methods, different parts of the alignments (only the catalytic part, the catalytic part plus the chitin-binding part, and the whole sequences minus the signal peptides) or using DNA or protein sequence (data not shown).

When a phylogenetic tree was made for the lectins, our clone, *Go-lec*, was classified as a lectin, as presented in Fig. 6. The other lectins on the same branch as *Go-lec* in the phylogenetic tree are *LECPRA3* from *R. pseudoacacia*, a lectin from *Lotus tetragonolobulus*, and *Blec* and *Nlec from P. sativum* (Fig. 6). Fig. 4 shows an alignment of the *Galega* lectin with lectins from *R. pseudoacacia, C. arietinum, L. tetragonolobus, P. sativum (PSL, Blec and Nlec), Arachis hypogaea* and *Psophocarpus tetragonololus.*

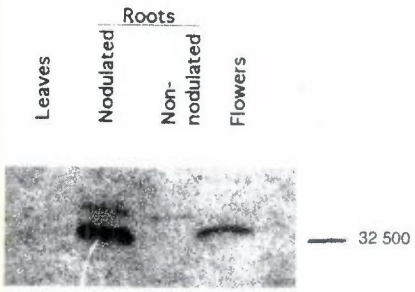

Figure 7. Western blot with protein prepared from leaves (of nodulated plants), nodulated roots, non-nodulated roots and flowers (from nodulated plants) of *Galega orientalis*.

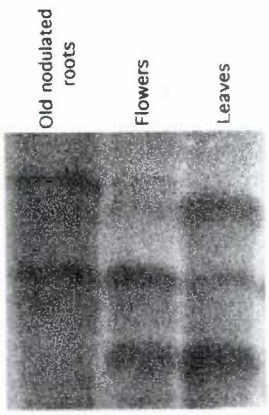

Figure 8. Chitinase activity gel with proteins isolated from old roots with nodules, flowers and leaves. At least four bands of different pI with chitinase activity can be seen.

Expression analysis

Chitinase expression was compared among different tissues of *G. orientalis* using Western blots. The antiserum was made against a class II chitinase with a size of 26 kDa. In nodulated roots, two clear bands are visible (Fig. 7), where one is very strong. In roots not inoculated with rhizobia, only one weak band is visible. In leaves a weak band is observed, whereas one strong band can be observed in flowers. The band from *G. orientalis* is larger than the protein the antibody was made against, and is therefore probably a class I or IV chitinase. The upper band was estimated to 35 kDa and the lower to about 32.5 kDa.

Tissue-specific chitinase activity against chitosan was analysed. In leaves three single bands can easily be distinguished. Flowers have four bands, two of them with almost the same size (Fig. 8). One of these bands also exists in leaves, and one in roots. Old roots are missing one band, which is strong in leaves and flowers. One of the chitinases seems to be active in all three tissues. The protein concentrations in the isolations from young roots, nodulated and non-nodulated, were too low to be detected on an activity gel.

After running RT-PCR with RNA isolated from nodulated and non-nodulated roots with primers binding to either one of the chitinase genes (*Go-cht1b*, AY253986) or the lectin gene, bands were visible both in nodulated and non-nodulated roots.

The results from the RT-PCR are presented in Tables 1 and 2. The two chitinases as well as the lectin genes were equally expressed in the nodulated and non-nodulated 5 days old plants, while the defensin gene was not expressed in either. As for the 30 days old plants, the lectin continued to be expressed in nodulated and non-nodulated plants, while the expression of both types of chitinases had disappeared in the non-nodulated plants. However, expression of the defensin gene was only detected in older non-nodulated plants.

4. Discussion

Chitinases

The classification of chitinases is summarised in Fig. 1. Three out of the four identified chitinase clones from *G. orientalis* could be classified as belonging to class I chitinases, whereas one was a class IV chitinase based on comparisons to previously published sequences. Clone *Go-chtIV* (AY253984) shows a high homology to class I but includes the four deletions, specific for class IV, thus classifying this sequence as a class IV chitinase (Fig. 3). Moreover, it has no vacuole signal, corresponding with the fact that most class IV-chitinases are extracellular (Lange et

al., 1996). The length of the hinge region is 5 amino acids longer in *Go-chtlb2* than in *Go-chtlb*. This fits the previous observations that the lengths of the hinge region are variable even among the different chitinases in the same species.

It is interesting that the sequences of the *Galega* chitinases do not differ significantly from the chitinases of other legumes, while *R. galegae* Nod factors have an unusual O-acetyl group not found in Nod factors from other legumes (Yang et al., 1999). This indicates that in addition, other components must be involved in the narrow host specificity between *G. orientalis* and *R. galegae*. The NodD protein, and the flavonoids from *G. orientalis* are important factors in this specificity (Suominen et al., 2003; Yang et al., 1999).

Phylogenetically *R. galegae* has been shown to be more closely related to *Agrobacterium* than the other rhizobia, and more distant from other rhizobia (Spaink et al., 1998; Terefework et al., 1998). Biochemical tests of the *Galega* chitinases and purified *R. galegae* Nod factors for degradation by the chitinases are needed to test their importance for host- microsymbiont specificity.

Four chitinase cDNA-clones were isolated in total. We have shown that there is a tissue-specific expression of chitinases of *Galega*. Chitinases are often expressed in flowers to protect the reproductive tissue. In the western blot (Fig. 7), there are two bands in the nodulated roots and one in flowers, while in the activity gel, there are more bands in 17 flowers than in nodulated roots. This is due to the fact that the two different methods will not identify the same chitinases. On the activity gel, basic proteins are separated, and all basic proteins with chitinase activity will be seen as bands. Some of these might be class III or V chitinases. The sequences of those are totally different to chitinases of class I, II and IV, making it unlikely that they should be recognised by a chitinase II antibody. The RT-PCR showed that one of the chitinases (AY253986) is expressed in both nodulated and non-nodulated roots (Table 2).

Lectins

The clone *Go-lec* was classified as a lectin. Vegetative lectins and seed lectins appear at two different branches in the tree. Our lectin gene ends up in the vegetative branch of the tree. The RT-PCR showed that our lectin gene is expressed in both nodulated and nonnodulated roots (Table 2). The *Go-lec* lectin lacks one of the two essential glycines (the last one, corresponding to Gly-246 in PSL), but not the other, as is the case in *LECRPA3* in *R. pseudoacacia*. A partial sugar-binding function of *Go-lec* thus cannot be excluded. PsNLEC1 is the major glycoprotein component in root nodules of *P. sativum*. It is not known if the proteins on this branch have any sugar binding activity (Dahiya et al., 1997). *Nlec* and *Blec* both lack two G that are essential for sugar binding in the pea seed lectin PSL (Dahiya et al., 1997). Those two essential carbohydrate-binding glycines in PSL (a seed lectin from *P. sativum*) are also missing in *PsNLEC1* (PSL Gly-128 and Gly-246 in Fig. 4) (Kardailsky et al., 1996). Vacuolar-localised lectins have an LQGD-sequence in the beginning of the amino acid sequence, which functions as a vacuolar target and where the D is very conserved (Law 1996). Our lectin has instead the sequence LQGS (Fig. 4, underlined) and thus cannot be concluded to have a vacuolar localization. This *Go-lec* may instead be targeted 18 to be secreted which would make it interesting as a candidate to bind rhizobia to the root surface. The only one partial lectin sequence previously published from *G. orientalis* (AJ234390) by Brewin and Kardailsky (1997) shares almost no homology with our lectin. sequence.

Other proteins

From the root library, a short sequence was isolated that when translated has a weak homology to the defensin family of proteins. Contrary to the RT-PCR expression of the tested lectin and chitinases, n93 is only expressed in 30 days old nodules.

Our n93 has eight cysteines in total, as in other defensins, but the C-terminal cysteine is not found at the same position as in other defensins. The cysteine-rich proteins found in nodules (NCRs, for nodule-specific cysteine rich) have a conserved signal peptide not observed in n93 (Mergaert et al, 2003, Graham et al., 2004). The NCR that Mergaert et al. (2003) found in *G. orientalis* show limited similarity to n93. n93 seems to be more similar to antifungal defensins and proteinase inhibitors

Table 2. Expression of genes (determined by RT-PCR) in 5 and 30 days old plants.

Gene/Plant	Chitinase class I	Chitinase class IV	Lectin	Defensin	Ubiquitin
5/not nodulated plant	+	+	+	–	+
5/nodulated plant	+	+	+	–	+
30/not nodulated plant	–	–	+	–	+
30/nodulated plant	+	+	+	+	+

+: presence of fragment, –: absence of fragment.

from plants and insects, which are involved in pathogenic defence. We suggest this gene to be involved in the defence of the root against pathogens.

Acknowledgements

SJFR, Swedish Council for Forestry and Agricultural Research and The Nordic Joint Committee for Agricultural Research are acknowledged for financial support. The help from the following people are gratefully acknowledged: Tomas Bryngelsson and Britt Gréen, Plant Science, Swedish University of Agricultural Science, Sweden; Niclas Olsson and Lena Carlsson, Cell- and Organic Biology, Lund University; Kristina Lindström and the members of her group, Department of Applied Chemistry and Microbiology, Katileena Lohtander, Department of Applied Biology, University of Helsinki, Finland.

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