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Review

Chitinases in root nodules

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Abstract The abundance of chitinases in plants is surprising in view of the fact that plants do not contain chitin. However, plant chitinases have been shown to play a role in defense, growth and developmental processes. They are also involved in plant-bacterial symbioses. Two groups of plants, legumes and actinorhizal plants, are able to enter root-nodule symbioses with nitrogen fixing bacteria, rhizobia and *Frankia* strains, respectively, and plant chitinases are involved in these interactions. None of these bacteria contain chitin in their cell walls but rhizobia produce chitinaceous signal factors. To find out whether symbiosis-related chitinases belonged to phylogenetically distinct subgroups, a phylogenetic analysis was performed including all chitinases of one dicot, Arabidopsis, and one monocot, rice. The results show that conserved class I- and class III-chitinases were recruited in both types of root nodule symbioses. Since no chitinaceous signal molecules are formed by *Frankia*, a role of chitinases in the control of microbial signaling is unlikely. Alternative roles of chitinases in root nodules are discussed.

Key words: Chitinase, *Frankia*, rhizobia, root nodule, actinorhiza.

Chitinases in plants

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 all true fungi, including fungi involved in arbuscular mycorrhiza (AM) and ectomycorrhiza (EM), and of the exoskeleton of insects and crustacean shells (Boller 1987; Salzer et al. 2000; Kasprzewska 2003).

Chitinases can be divided into two categories: exochitinases, which show activity only for the non-reducing end of the chitin chain; and endochitinases, which hydrolyse internal β-1,4-glycoside bonds (Kasprzewska 2003). Based on their physicochemical properties and enzymatic activity, several nomenclature systems exist for chitinases: as glycoside hydrolases (families 18 and 19), as pathogenesis-related (PR) proteins, as chitinase classes, and as gene families (Yokoyama and Nishitani 2004). Classification based on sequence comparisons structure led to a total of five different classes of chitinases (seven in other classification systems). Plant chitinases are organized in five classes numbered from I to V (Neuhaus et al. 1996). Chitinases from classes I, II and IV belong to glycoside hydrolase family 19, whereas classes III and V are comprised of glycoside hydrolase family 18 chitinases (Passarinho and de Vries 2002). The classification is further complicated by the fact that members of glycoside hydrolase family 18 that group with class III chitinases, can be devoid of chitinase activity and represent xylanase inhibitor proteins (Durand et al. 2005).

The abundance of chitinases in plants is surprising in view of the fact that plants do not contain chitin. Chitinases have been localised in all plant organs and -tissues, both in the apoplast and in the vacuole, with different molecular structures and substrate specificities (reviewed by Kasprzewska 2003). Initially, plant chitinases were considered as pathogenesis-related (PR) proteins involved in the defense against fungal pathogens (reviewed by Graham and Sticklen 1994), and several lines of evidence showed increased resistance to fungal pathogens as result of the transgenic expression of chitinases (Jach et al. 1995; Ebel 1998; Maximova et al. 2006). However, further investigations have also shown that chitinases play a role in growth and development processes such as pollination, senescence, root and root nodule development, seed germination and somatic embryogenesis (Collinge et al. 1993; Goormachtig et al. 1998; Helleboid et al. 2000; Regalado et al. 2000; Kasprzewska 2003).

Root nodule symbioses

Only prokaryotes can reduce air dinitrogen (N_2) to ammonium. Two groups of plants, legumes and

actinorhizal plants, are able to form root-nodule symbioses with N_2-fixing bacteria, rhizobia and *Frankia*, respectively. In these symbioses, the bacteria reduce N_2 to ammonium while being hosted inside plant cells in special organs, the root nodules. Due to the O_2 sensitivity of the enzyme responsible for N_2 fixation (nitrogenase), this process can only take place when nitrogenase is protected from O_2; nevertheless, the high amount of ATP required for the reduction of N_2 has to be provided by respiration. In nodules, structural and physiological mechanisms are combined to solve this dilemma.

Legume and actinorhizal symbioses are evolutionary related (Soltis et al. 1995) and share many common aspects, especially regarding the infection process and nodule functioning, despite the remarkable differences between the microsymbionts (Pawlowski and Bisseling 1996; Pawlowski and Sprent 2008). Biological N_2 fixation makes up nearly 50% of N_2 fixation worldwide, and symbiotic fixation accounts for a large part of biologically fixed N_2 (Bezdicek and Kennedy 1998).

Legume symbiosis

Rhizobia–unicellular Gram-negative soil bacteria–induce nodules on the roots of legumes and one non-legume *Parasponia* sp. (Ulmaceae; Mylona et al. 1995). Mature legume nodules are stem-like organs with peripheral vascular system and infected cells in the inner tissue. The bacteria are hosted inside infected nodule cells, where they produce nitrogenase and fix nitrogen.

During legume nodule induction, plant roots secrete flavonoids as a response to nitrogen deprivation. These flavonoids induce the transcription of rhizobial *nod* (*nol, noe*) genes. Proteins encoded by these genes catalyze the synthesis of specific lipochitooligosaccharides, the so-called Nod factors, which induce the early steps of nodule formation and are the main determinants of host-specificity in rhizobial symbioses. Due to their chitin backbone, several chitinases have been found to be capable to differentially hydrolyse Nod factors (Minic et al. 1998; Perret et al. 2000).

Nodule formation starts with infection which can occur in two ways, (a) intracellularly and (b) intercellularly. During intracellular infection, Nod factors induce the deformation of root hairs, the formation of pre-infection thread structures (PITs) in cortical cells, and cell divisions in the cortex that later will give rise to the nodule primordium. Concomitantly, rhizobia enter the plant root within an infection thread formed in a curled root hair, embedded in the infection thread matrix (Mylona et al. 1995). When infection threads reach nodule primordium cells, the bacteria are stably intracellularly accomodated in these cells in a complete endocytotic process, surrounded by peribacteroid membranes derived from the plasma membrane of the infected cell. Within the infected cells, they differentiate into their N_2-fixing form, the bacteroids. During intercellular infection, bacteria in most cases enter the roots via cracks at the junctions of emerging lateral or adventitious roots. In some cases, infection threads can be formed later, while in other cases, the bacteria move through the apoplast, but finally, the bacteria are stably intracellularly accomodated as bacteroids within infected cells (Sprent and James 2007; Pawlowski and Sprent 2008).

Actinorhizal symbioses

In the case of actinorhizal symbioses, *Frankia*–filamentous, branching, Gram-positive actinomycetous soil bacteria–induce nodules on the roots of dicotyledonous plants from eight different families, mostly trees or woody shrubs, collectively called actinorhizal plants. Like rhizobia, *Frankia* strains produce nitrogenase, but in contrast with most rhizobia, *Frankia* strains can fix nitrogen in the free-living state, not only under microaerobic, but also under aerobic conditions. In the latter case, they form special vesicles at the ends of hyphae or short side hyphae wherein nitrogenase is protected from O_2 (reviewed by Benson and Silvester 1993). Mature actinorhizal nodules are coralloid organs composed of multiple lobes, each of which represents a modified lateral root without root cap, a superficial periderm and infected cells in the expanded cortex (Pawlowski and Bisseling 1996).

So far, the signal exchange during actinorhizal nodule induction is poorly understood. Flavonoids in the root exudates have been suggested to activate the synthesis of *Frankia* Nod factors (Prin and Rougier 1987; van Ghelue et al. 1997; Laplaze et al. 1999). Like rhizobia, *Frankia* strains can enter the roots of their host plants either intracellularly via root hairs, or intercellularly, and the mode of infection depends on the host plant species. Analogous to intracellular infection described for rhizobia, *Frankia* culture supernatants contain a factor of unknown chemical nature that induces the deformation of root hairs. When a hypha is trapped in a root hair curl, an infection thread-like structure (ITL) develops by which the hypha enters the plant root embedded in a cell wall-like matrix, the equivalent of the infection thread wall in legume nodules. Actinorhizal nodule primordia are formed in the root pericycle like lateral root primordia. When an ITL reaches the nodule primordium, primordium cells are infected by intense branching of the ITLs inside the cell. During intercellular infection, *Frankia* hyphae enter the root by penetration between epidermal cells and colonize the root cortex intercellularly. In contrast with rhizobia, *Frankia* does not depend on gaps in the root epidermis for infection (Pawlowski and Bisseling 1996).

Rhizobial Nod factors

The role and structure of bacterial signal factors have been studied with great detail for legume-rhizobia symbioses (Dénarié et al. 1996; Long 1996). Nod factors consist of an oligomeric chitin backbone of β-1,4-linked *N*-acetyl-D-glucosaminyl residues, N-acylated at the non-reducing-terminal residue. The N-acylation can either represent a 'common' fatty acid like C18:1 or a (poly)unsaturated fatty acid (Dénarié et al. 1996; Kamst et al. 1998; reviewed by D'Haeze and Holsters 2002). Nod factor synthesis depends on the expression of rhizobial *nod*, *nol* and *noe* genes (D'Haeze and Holsters 2002). The synthesis of the 'common' acylated chitin oligosaccharide backbone of the Nod factor depends on the 'common' NodA, NodB and NodC proteins. These proteins represent oligochitin GlcN *N*-acyltransferase (NodA), oligochitin GlcNAc de-*N*-acetylase (NodB) and UDP-GlcNAc transferase (NodC). NodB de-*N*-acetylates a terminal GlcNAc residue on a chitin oligomer (chitobiose, chitotriose and chitotetrose). NodA catalyzes the transfer of the fatty acyl group from an acyl carrier protein (ACP) to the de-*N*-acetylated terminal GlcN residue on the chitin oligomer. NodC is assumed to be involved in the synthesis of the chitin oligomer intermediate (Carlson et al. 1994). Both NodB and NodA do not show sequence similarity to analogous enzymes involved in lipid-A synthesis. Thus, it is likely that Nod factor biosynthesis involves a novel pathway which does not rely on enzymes that are required for the synthesis of other essential molecules. Nod factors represent the basis of host specificity and many different *nod*, *nol* and *noe* genes are involved in the various modifications of Nod factor structure (Carlson et al. 1994).

Rhizobial Nod factors are not only involved in nodule induction, but can also play a role in later stages of nodule development. Sharma and Signer (1990) and D'Haeze et al. (1998) showed that *nodA* is transcribed in symbiotic bacteroids in the symbioses of *Medicago sativa* and *Sesbania rostrata*, respectively, although Schlaman et al. (1991) found that this was not the case for bacteroids in pea nodules. Detailed analyses of the *S. rostrata* symbiosis revealed that symbiotic transcription of *nodA* was not essential for the symbiosis, but prevented premature senescence of nodules (Gao et al. 2001).

Chitinases in root nodules

Several chitinase genes have been found to be expressed specifically in root nodules, or to be expressed at elevated levels in nodules compared to roots, or to be present in the root exudate (Table 1). While it is possible that root nodule symbioses evolved from a parasitic interaction, neither rhizobial nor *Frankia* cell walls contain chitin, thus excluding a role for chitinases in the control of the propagation of the microsymbiont within the plant. However, a possible function assigned to chitinases in symbioses is the control of the infection process in legumes. Degradation of the chitin backbone of bacterial Nod factors by chitinases leads to their inactivation, thus reducing the efficiency of root nodule formation (reviewed by Kasprzewska 2003). For example, certain chitinase isozymes are specifically induced in soybean nodules and near aborted infection threads in the interaction between alfalfa and *Sinorhizobium meliloti* (Salzer et al. 2004). Consistently, in soybean roots, rhizobial Nod factors induced an increase in chitinolytic activity, suggesting a role in an early and perhaps transient feedback process (Xie et al. 1999).

The continued activity of *nod* gene expression in developing or mature nodules might lead to the assumption that also in these stages, chitinases could control infection by Nod factor degradation as suggested by Goormachtig et al. (1998). D'Haeze et al. (2002) suggested a role for chitinases in Nod factor perception, but this is unlikely as Nod factor receptors have meanwhile been identified and shown to represent LysM domain receptor kinases (Limpens et al. 2003; Madsen et al. 2003). Furthermore, in actinorhizal symbioses where chitinases were also expressed in mature nodules, no equivalents of rhizobial Nod factors have been identified. While the presence of a root hair-deforming factor could be shown (van Ghelue et al. 1997), this factor was structurally different from rhizobial Nod factors (Cérémonie et al. 1998; 1999), and apparently not degraded by chitinases (Cérémonie et al. 1999). Furthermore, no homologues of the common *nod* genes *nodA* and *nodC* were found in the three sequenced *Frankia* genomes (Normand et al. 2007), making it extremely unlikely that *Frankia* strains can produce molecules resembling rhizobial Nod factors. Hence, there must be other roles for chitinases in developing or mature nodules than the degradation of rhizobial Nod factors.

A role of chitinases in the defense of nodules against fungal soil pathogens would only be likely if nodule-specific chitinases were present specifically in external nodule tissues. However, this was not the case for several nodule chitinases examined, e.g. from *S. rostrata* (Goormachtig et al. 1998; 2001) and from the actinorhizal plants *Elaeagnus umbellata* (Kim and An 2002) and *Casuarina glauca* (Fortunato et al. 2007). Interestingly, most nodule-specific chitinases belonged to class III.

Roles of chitinases in plants

The role of chitinases in the defense against fungal pathogens is undisputed (Kasprzewska 2003). What

Table 1. Chitinases from roots and/or nodules of root nodule-forming plants. Simplified names have been given to those chitinases for which the full sequence information is available, for use in Figures 1A and 1B; these names are given in brackets below the gene/protein name. The subcellular localization was determined using PSORT (http://psort.nibb.ac.jp/).

Species	Gene/Protein Name	Class	Subcellular Localization	Expressed in Organs/Cell types	Reference
Sesbania rostrata	*SrChi13* (SrIIIa)	III	apoplastic	immature nodules, around infection pockets in infection centers, around the developing nodule and its vascular bundles, and in uninfected cells of the central tissue	Goormachtig et al. (1998)
	SrChi24 (SrIIIb)	III	apoplastic	flowers, seedlings, immature root nodules; outermost cell layers of nodules	Goormachtig et al. (2001)
Medicago sativa	CHIT24	I	unknown	seedling roots	Minic et al. (1998)
	CHIT36	I	unknown	seedling roots	Minic et al. (1998)
Medicago truncatula	*MtChitI* (MtI)	I	mitochondrial outer membrane or apoplastic	pathogen-induced in roots	Salzer et al. (2000)
	MtChitIII-1 (MtIIIa)	III	apoplastic	pathogen-induced in roots	Elfstrand et al. (2005)
	MtChitIII-3 (MtIIIc)	III	apoplastic or vacuolar	arbuscular mycorrhizal roots	Elfstrand et al. (2005)
	MtChitIII-4 (MtIIId)	III	apoplastic or vacuolar	arbuscular mycorrhizal roots	Elfstrand et al. (2005)
	MtChitV (MtIV)	IV	apoplastic or peroxisomal	nodulated or pathogen-treated root systems	Salzer et al. (2004)
	MtChitV (MtIV)	V	plasma membrane or peroxisomal	nodulated root systems	Salzer et al. (2004)
Vicia faba	*NVf32* (VfIIIa, VfIIIb)	III	peroxisomal or nuclear	nodules; nitrogen-fixation zone	Perlick et al. (1996)
Elaeagnus umbellata	*EuNOD-CHT1* (EuIa)	Ia	apoplastic or vacuolar	nodules, root tips, leaves; in nodules in meristem, outer cortex, uninfected cortical cells of fixation zone	Kim and An (2002)
	EUNOD-CHT2 (EuIb)	Ib	apoplastic	roots, nodules, lowly in leaves; in nodules in nitrogen-fixing infected cells and vascular system, senescence zone	Kim and An (2002)
Casuarina glauca	*CgChi3* (CgIII)	III	apoplastic or vacuolar	nodules, infected and uninfected cortical cells, vascular tissue	Fortunato et al. (2007)
Casuarina glauca	*CgChi1* (CgI)	I	vacuolar	roots and nodules	P. Santos, A. Fortunato and A. Ribeiro, unpublished (EU346700)

is less clear is the biochemical basis for the role of chitinases in plant development. Furthermore, a conserved chitinase sequence does not necessarily denote chitinase activity. As mentioned earlier, a subgroup of class III family 18 endochitinases has no enzymatic chitinase activity and instead works as xylanase inhibitor proteins (XIPs; McLauchlan et al. 1999). Xylan is the predominant hemicellulose in the plant cell walls and the second most abundant polysaccharide on earth. Analyses showed that out of a range of fungal and bacterial endoxylanases tested for XIP-sensitivity, all those of fungal origin were inhibited by XIP, with the sole exception of one endoxylanase from *Aspergillus aculeatus* (Flatman et al. 2002; Juge et al. 2004). Hence, the function of XIPs is likely to be linked to pathogen defense, not to plant cell wall modifications. Plant chitinases evolve rapidly, suggesting that they are critical in the coevolution of plants and pathogens (Bishop et al. 2000). Therefore it is likely that XIP proteins evolved from chitinases whose synthesis was already triggered by fungal attack (Durand et al. 2005). This way, the existing signal recognition and expression-regulation pathways could have been retained (Beliën et al. 2006). So far, XIPs have only been isolated from cereals, despite until recently xylanase inhibitors have been believed to be absent in rice (Goesaert et al. 2004), which is consistent with the fact that cereal cell walls contain arabinoxylan as structural components (Raedschelders et al. 2004).

It has long been suggested that chitinases may regulate plant growth and development by modifying polysaccharides attached to proteins, or by generating or degrading signal molecules (reviewed by Kasprzewska 2003; D'Haeze and Holsters 2002). Thus, rhizobial Nod factors might represent imitations of endogenous plant signal molecules. Several studies support this hypothesis. E.g., the expression of the deacetylase gene *nodB* and

the acyl transferase gene *nodA* in tobacco, singly or in combination, affected plant growth and development, indicating that plants contain chitin oligomers that NodA and NodB could modify (Schmidt et al. 1993). Furthermore, in *Daucus carota*, chitinases were shown to be able to cleave polysaccharides on arabino-galactan proteins (AGPs) *in vitro* and to co-localize with AGPs in developing seeds (van Hengel et al. 1998; 2001), which in turn suggests that AGPs which have been implicated in the regulation of cell differentiation, could be the endogenous substrates for plant chitinases. In embryogenic cultures of both *D. carota* and *Picea abies*, bacterial Nod factors can substitute for chitinases in their effect on early somatic embryo development (De Jong et al. 1992; Egertsdotter and von Arnold 1998; Dyachok et al. 2005). An acidic class III endochitinase (glycoside hydrolase family 18) from cowpea (*Vigna unguiculata*) was found to represent a protein that is required for cell wall loosening during acid growth (yieldin; Okamoto-Nakazato et al. 2000a; 2000b). Sasaki et al. (2006) compared the substrate specificity of class I and class III chitinases from rice and found that the class III enzyme might act towards an endogenous complex carbohydrate containing a GlcNAc residue, but not against a GlcNAc oligomer or polymer, while the substrate of the class I enzyme was probably a consecutive GlcNAc sequence, maybe from the cell wall of a fungal pathogen.

Like class III enzymes, also chitinases from other classes seem to be involved in the modulation of cell wall development. The mutation of AtCTL1, a glycoside hydrolase family 19 class II endochitinase from Arabidopsis, led to defects in cell wall synthesis causing increased ion leakage and hypersensitivity to high salt stress and osmotic stress (Kwon et al. 2007).

Are nodule chitinases from different symbiotic plants phylogenetically related?

Yokoyama and Nishitani (2004) have performed a phylogenetic analysis of chitinase proteins from rice and Arabidopsis and shown that they form two distinct subfamilies. Interestingly, the second subfamily, which also includes the cowpea yieldin mentioned above, contained only one member of Arabidopsis and 27 rice chitinases, while the first subfamily contained about equal numbers of rice and Arabidopsis chitinases. The size of the yieldin subfamily in rice suggested that roles of yieldin-homologues had diversified in this species, maybe related to the mode of cell-wall expansion in monocotyledonous versus dicotyledonous plants.

Based on this information, we performed a new phylogenetic analysis of all Arabidopsis and rice chitinase sequences from Yokoyama and Nishitani (2004) that contained a more or less complete glycoside hydrolase 18 or 19 domain. In this analysis, all full size sequences of chitinases studied in roots and/or nodules of legumes and actinorhizal plants were included (Figure 1). Two nodule-specific chitinase homologues from broad been (*NVf32a, b*) were found to be expressed in the nitrogen-fixing zone of the inner tissue of the nodule (Perlick et al. 1996) but since the encoded proteins do not contain a full glycoside hydrolase (GH) 18 domain and their highest homology is to Narbonins (2S seed storage proteins), for which no enzymatic function could be detected (Steffens et al. 1997), *NVf32a* and *b* were not included in the phylogenetic analysis.

Due to their diverse nature, members of the GH 18 (classes III and V; Figure 1A) and of the GH 19 family (classes I, II and IV; Figure 1B) were analysed separately. The results showed that the yieldin-containing subfamily described by Yokoyama and Nishitani (2004) was now restricted to a group of dicot class III chitinases including yieldin and one rice sequence, P0656C04.02. The only class III chitinase from Arabidopsis fell in this group (Fig. 1A). The rest of class III chitinases included all XIP-like rice sequences. All class III chitinases from roots/nodules, including the chitinases from *M. truncatula* that are induced specifically by arbuscular mycorrhization, but not by nodulation (*MtChitIII-1* (MtIIIa), *MtChiIII-3* (MtIIIc) and *MtChiIII-4* (MtIIId); Salzer et al. 2004) fell into the the yieldin group, indicating that they might fulfill a conserved function in determining cell wall flexibility. It would be tempting to assume that the function of these chitinases is related to the stable intracellular accomodation of microsymbionts. However, the expression patterns of the nodule-specific chitinases from this group are quite diverse, and none of them is expressed exclusively in infected cells. E.g., *SrChi13* (SrIIIa) from the leguminous tree *S. rostrata* was only expressed in uninfected cell types and was suggested to be involved in protecting cells from rhizobial invasion (Goormachtig et al. 1998), while the expression of *SrChi24* (SrIIIb) from the same plant was confined to the outer layers of the nodule where no infected cells are present and a function in the defense against exogenous pathogens was most likely (Goormachtig et al. 2001). Both chitinase genes were not expressed in mature nodules. In contrast, *CgChi3* (CgIII) from the actinorhizal tree *Casuarina glauca* was expressed in infected and uninfected cells of the nodule cortex throughout nodule development, and also in the nodule vascular system (Fortunato et al. 2007). Moreover, not all nodule-specific chitinases belong to class III: the nodulation-specific class V chitinase from *M. truncatula*, *MtChitV* (MtV) whose expression pattern in nodules was not examined (Salzer et al. 2004), showed highest homologies with pathogenesis-related chitinases from tobacco (Heitz et al. 1994; Melchers et al. 1994). In the GH 18 phylogenetic tree, MtV grouped with a rice, not with an Arabidopsis class V chitinase

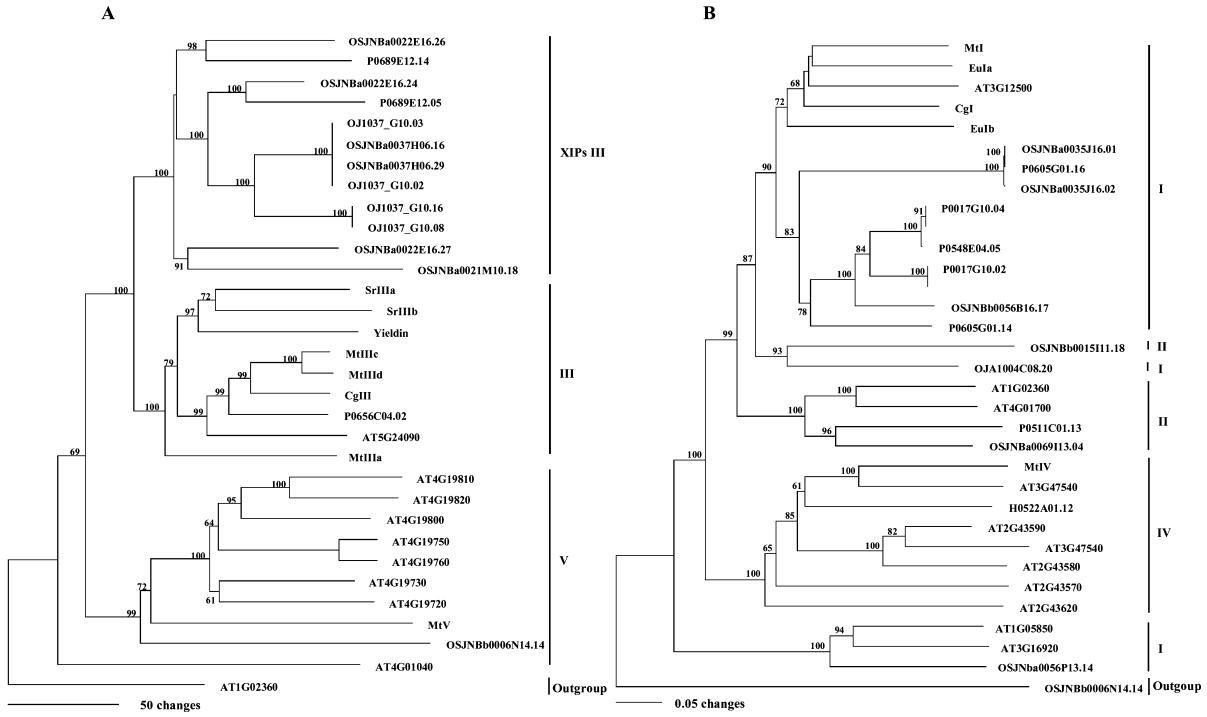

Figure 1. (A) Phylogenetic tree for the GH 18 chitinase family. (B) Phylogenetic tree for the GH 19 chitinase family. Complete amino acid sequences were retrieved from the supplementary data available online by Yokoyama and Nishitani (2004), www.ncbi.nlm.nih.gov and http://ricegaas.dna.affrc.go.jp/. Cowpea yieldin protein (AB028025) is included in the tree. Outgroups: (A) Arabidopsis class II chitinase (AT1G02360); (B) Rice class V chitinase (OSJNBb0006N14.14). Sequence analysis was performed using ClustalX for multiple alignment (Thompson et al. 1997) and the phylogenetic trees were estimated by neighbor-joining analysis using the software PAUP* 4.0b10 (PPC/Altivec) for Macintosh (Swofford 1998; Florida State University, Miami, FL, USA). Bootstrap analysis with 1000 bootstrap replications using the neighbor-joining search option of the program PAUP* 4.0b10 was carried out to test the robustness of the internal branches.

(GH18-OSN14.14; Figure 1A).

All class I chitinases (*EuNOD-CHT1* (EuIa) and *EuNOD-CHT2* (EuIb); Kim and An 2002; *CgChi1* (CgI); P. Santos, A. Fortunato and A. Ribeiro, unpublished) known to be expressed in nodules, as well as MtI from *M. truncatula* (Salzer et al. 2000) were grouping with a basic class I chitinase from the GH 19 family that seemed to be expressed at highest levels in Arabidopsis roots (AT3G12500; www.tigr.org; Figure 1B). Like class III chitinases, class I chitinases seemed to have diversified in rice more than in Arabidopsis. This difference in diversification may not be related to the monocot/dicot distinction as suggested by Yokoyama and Nishitani (2004), but to the fact that Arabidopsis is not able to enter an arbuscular mycorrhizal (AM) symbiosis whereas rice is. AM symbioses have been suggested as the evolutionary precursor of root nodule symbioses (Kistner and Parniske 2002), and some chitinase genes are known to be induced specifically in arbuscular mycorrhizal symbioses (Blee and Anderson 1996; Salzer et al. 2000). Consistent with studies from Gomez et al. (2002), Figure 1B suggests that class IV chitinases have evolved from class I and class II chitinases. The *Mtchit4* (MtIV) chitinase gene that could be induced by nodulation as well as pathogen infection (Salzer et al. 2004), clustered with a group of homologues of pathogen-induced chitinases as described in the original publication.

Since root/nodule class III chitinases from legumes and actinorhizal plants were grouping together, and so did class I chitinases, it seems unlikely that either chitinase group evolved to act on rhizobial Nod factors.

The conclusions to be drawn from this analysis for the phylogeny of chitinase classes in general, are limited due to the selection of sequences. At any rate, according to Figure 1B, neither class I nor class II chitinases form a monophyletic group, while class IV chitinases do. These results support earlier studies from Araki and Torikata (1995) who did not find a monophyletic origin for class I and class II. They also support the results an analysis involving all chitinase sequences from flowering plants available at the time performed by Hamel et al. (1997) who could not find a monophyletic origin for class II chitinases but established a monophyletic origin for class IV chitinases which according to Wiweger et al. (2002) separated from class I/class II chitinases before the separation of gymnosperms and angiosperms, ca. 300 Mio years ago.

Conclusions

Chitinases play a role in pathogen defense either by chitin degradation (chitinolytic activity) or by xylanase inhibition (no chitinolytic activity). While the former effect is well studied in monocots and dicots, the latter has only been described for monocots as arabinoxylans represent a major component of the cell walls of Gramineae, but not of dicots. The role of chitinases in development is ascribed to modification of the glycosylation of AGPs, or alternatively in the case of class III chitinases with strong homology to yieldin, to an effect on the yield threshold of cell walls via an unknown mechanism.

Several class III chitinases expressed in roots and/or nodules from legumes or actinorhizal plants were found to belong to the yieldin group. A comparison of their expression patterns did not allow any conclusion regarding a common function. Three of the class I chitinases from roots or nodules—two from actinorhizal plants and one, MtI, from a legume—were also clustering together. Nod factor-degrading chitinases from legumes have mostly been characterized on the level of enzyme activity, not on the sequence level; nevertheless, some class I chitinases have been found to be able to degrade Nod factors (reviewed by Perret et al. 2000). The close relationship between the pathogen- and nodulation-induced MtI and two chitinases from actinorhizal plants that react to structurally different signal molecules (Cérémonie et al. 1999) argues against a specialization of MtI for Nod factor degradation.

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