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INVITED REVIEW ARTICLE

Seed chitinases

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Abstract

Specific chitinases accumulate in seeds of many species as part of their normal developmental programme. Some chitinases can also be induced in developing and germinating seeds in response to microbial attack. All known seed isoforms belong to classes I, II, IV and VII, which are encoded by *Chia* genes, as well as to the more divergent class III encoded by *Chib* genes. The study of seed-specific chitinases has contributed significantly to current knowledge of this ubiquitous protein family, including antifungal properties, structure, specificity and catalytic mechanism. Indeed, the first plant chitinase for which a three-dimensional structure was solved had been isolated from barley seeds. Moreover, the finding that a chitinase could rescue a somatic embryo mutant was the first evidence of a non-defensive function. Several lines of evidence have substantiated the biotechnological potential of chitinases to counter plant fungal disease. The recent identification of several seed and fruit chitinases as major panallergens should be taken into account when selecting the genes to be introduced into food crops.

Keywords: chitinase, seeds, antifungal proteins, PR-proteins, latex-fruit syndrome, glycosyl hydrolases, somatic embryogenesis

Introduction

Chitin, an insoluble β(1→4)-linked *N*-acetyl-D-glucosamine polysaccharide, occurs in a wide range of organisms. It serves a structural role in fungal cell walls and also in arthropod cuticles, including those of insects, nematodes and crustaceans. However, chitin has not been found in higher plants or in mammals. Nevertheless, plants (and mammals) produce a wide array of chitinases (EC 3.2.1.14) homologous to those found in chitin-containing organisms. Plant chitinases constitute a ubiquitous and heterogeneous family, members of which have been grouped into 11 different classes, according to their primary structure (Flach *et al*., 1992; Collinge *et al*., 1993; Raikhel *et al*., 1993; Sahai and Manocha, 1993; Stintzi *et al*., 1993; Neuhaus, 1999). A large body of evidence has substantiated the notion that the major natural role for these enzymes is defence, without excluding other functions in plant development and growth.

Chitinase expression is often induced by microbial attack and, in fact, many chitinases have been classified as pathogenesis-related proteins of the PR-3, PR-4, PR-8 and PR-11 families (Bol *et al*., 1990; Neuhaus *et al*., 1996; Fritig *et al*., 1998; Neuhaus, 1999). The *in vitro* antifungal activity of chitinases, either alone or in combination with other PR proteins, has been known for 15 years (Schlumbaum *et al*., 1986; Broekaert *et al*., 1988; Mauch *et al*., 1988; Boller, 1993). Chitinases are also synthesized in response to oligosaccharides from microbial or plant cell walls (elicitors) and after treatments with defence regulators, such as ethylene and salicylic acid (Boller, 1988; Fritig *et al*., 1998). Expression of chitinases in transgenic plants has substantiated their antifungal potential (Broglie *et al*., 1991; Vierheilig *et al*., 1993; Zhu *et al*., 1994; Grison *et al*., 1996). Finally, co-expression of barley chitinase and other defence proteins in transgenic tobacco has provided evidence for enhanced quantitative resistance against fungal disease (Jach *et al*., 1995).

Some chitinases are expressed only at well-defined developmental stages, such as seed maturation or flower and fruit development. These so-called constitutive chitinases have received much less attention than their stress-inducible homologues, despite the fact that their expression is typically restricted to non-vegetative organs (Neale *et al*., 1990;

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Leah *et al*., 1991; Collada *et al*., 1992; Cordero *et al*., 1994; Takakura *et al*., 2000). The first experimental evidence of a non-defensive role was obtained for a glycosylated chitinase that was able to rescue a carrot somatic embryo mutant (de Jong *et al*., 1992). More recently, the finding that Nod factors are substrates for plant chitinases has led to the proposal that isoforms synthesized in legume roots act as modulators of the plant–*Rhizobium* interaction (Staehelin *et al*., 1994; Schultze *et al*., 1998; Ovtsyna *et al*., 2000). In addition, during cold acclimation several freezing-tolerant plants accumulate specific chitinases, β-1,3-glucanases and thaumatin-like proteins in their vegetative tissues (Zhu *et al*., 1993; Hon *et al*., 1995; Gatschet *et al*., 1996; Ergon *et al*., 1998; Yeh *et al*., 2000). Antifreeze activity has been demonstrated for some of these proteins, such as the cold-inducible 35 kDa chitinase from winter rye leaves (Hon *et al*., 1995; Hiilovaara-Teijo *et al*., 1999). The cold-induced isoforms also show chitinolytic activity. Other lines of evidence support the hypothesis that chitinases have a role in plant growth (Patil and Widholm, 1997; Rohrig *et al*., 1995).

Compared to their vegetative counterparts, seed chitinases have been largely neglected. This is probably due to the fact that the former enzymes have been associated with defensive responses against pathogen attack or other types of stress. However, as we show in this review, many studies carried out with seed-specific chitinases have contributed significantly to our present knowledge of this important protein family. For recent comprehensive reviews on plant chitinases, the reader is referred to Neuhaus (1999) and Jollés and Muzzarelli (1999).

Classification

Plant chitinases can be classified into four major families, encoded by *Chia*, *Chib*, *Chic* and *Chid* genes (Neuhaus *et al*., 1996; Neuhaus, 1999). Different classes have been defined within each family, depending on sequence similarities and the presence of an N-terminal cysteine-rich domain, usually referred to as hevein-like domain or chitin-binding domain (CBD). When present, the CBD is separated from the catalytic domain by a hinge region, variable in length and amino-acid sequence. All known seed chitinases are encoded by either *Chia* or *Chib* genes (Fig. 1). *Chia* genes encode enzymes belonging to classes I, II, IV and VII (*Chia1*, *Chia2*, *Chia4* and *Chia7* genes, respectively). Only those enzymes of classes I and IV possess a CBD. While the four classes are all related, the catalytic domains of class I and II enzymes are particularly similar. Class IV and VII enzymes are smaller in size because of several deletions. Regarding the evolution of this family, it has been proposed that an ancestral *Chia* gene (class II) gave rise to class I chitinases by incorporating a CBD-encoding gene. Class I and II enzymes then evolved into classes IV and VII, respectively, through different pathways (Shinshi *et al*., 1990; Araki and Torikata, 1995; Hamel *et al.,* 1997). All seed chitinases encoded by *Chib* genes belong to class III. These chitinases are significantly different in structure and action from the other seed enzymes.

Characterization and phylogenetic relationships

The presence of seed-specific chitinases has not been thoroughly investigated in the plant kingdom. Different seed isoforms have been described in a number of plant species, belonging to phylogenetically distant families such as *Apiaceae*, *Cucurbitaceae*, *Fabaceae*, *Poaceae*, *Fagaceae* and *Pinaceae*. Most of these enzymes are induced as part of the seed developmental programme, with mRNA synthesis typically beginning at early or mid-maturation stages (Leah *et al*., 1991; Huynh *et al*., 1992; Yeboah *et al*., 1998). Some isoforms are also induced in developing and germinating seeds in response to microbial infection (Cordero *et al*., 1994; Ignatius *et al*., 1994; Wu *et al*., 1994; Caruso *et al*., 1999; Ji *et al*., 2000). It now seems clear that these pathogen-responsive chitinases are different from those described in leaves or roots (Majeau *et al*., 1990; Kragh *et al*., 1993; Cordero *et al*., 1994; Ignatius *et al*., 1994; Krishnaveni *et al*., 1999). Within seeds, chitinases have been reported to accumulate in different locations, including storage tissues (cotyledons and endosperm), aleurone, embryo and husks (Swegle *et al*., 1989, 1992; Jensen, 1994; Leah *et al*., 1994; Wu *et al*., 1994; Gijzen *et al*., 2001; Taira *et al*., 2001). The genes encoding enzymes present in mature seeds are active during the first 2 or 3 days of the germination process. At the same time, transcription of new chitinase genes is initiated (Leah *et al*., 1991; Huynh *et al*., 1992; Swegle *et al*., 1992). While the phytohormones abscisic acid and gibberellic acid do not seem to control chitinase expression in seeds (Leah *et al*., 1991; Wu *et al*., 1994), both ethylene-dependent and -independent pathways regulate their expression during pea seed germination (Petruzzelli *et al*., 1999). On the other hand, some genes for class IV enzymes, upregulated during somatic embryogenesis (de Jong *et al*., 1992; Dong and Dunstan, 1997), are also expressed during normal seed development (van Hengel *et al*., 1998).

Since the first well-characterized plant chitinase was isolated from wheat germ (Molano *et al*., 1979), a number of chitinases have been purified from seeds, particularly from cereal crops. No clear patterns emerge when comparing enzyme classes across species, even when only major isoforms are

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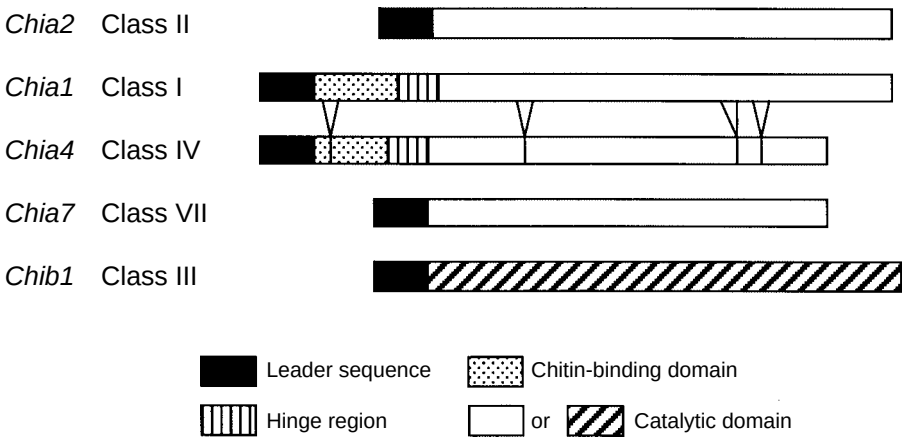

Figure 1. Schematic structure of the different chitinases found so far in seeds (classes I, II, III, IV and VII). These enzymes are synthesized as pre-polypeptides with an N-terminal signal sequence necessary for entry into the lumen of the endoplasmic reticulum (Chrispeels, 1991).

considered. For example, the most abundant chitinases in barley and rye belong to classes I and II, while in maize they belong to classes I and IV (Leah *et al.*, 1991; Kragh *et al.*, 1991, 1993; Huynh *et al.*, 1992; Swegle *et al.*, 1992; Yamagami and Funatsu 1993a, b, 1994; Wu *et al.*, 1994). The major enzyme types can also differ within the same genus. This becomes evident when comparing *Castanea sativa*, with one class I and two class II enzymes, and *C. crenata*, with only class II chitinases (Collada *et al.*, 1992, 1993). In contrast to these findings, SDS-PAGE analyses of seed chitinases from different maize, sorghum and wheat cultivars revealed little intraspecific variability (Darnetty *et al.*, 1993). Besides the above species, chitinases have also been purified from the seeds of *Benincasa hispida* (Shih *et al.*, 2001), *Canavalia ensiformis* (Hahn *et al.*, 2000), *Cucumis sativus* (Majeau *et al.*, 1990), *Glycine max* (Wadsworth and Zikakis, 1984; Yeboah *et al.*, 1998; Gijzen *et al.*, 2001) and *Sorghum bicolor* (Krishnaveni *et al.*, 1999). In addition to their hydrolytic action on chitin polymers, some of these enzymes also have lysozyme activity *in vitro* (Roberts and Selitrennikoff, 1988; Majeau *et al.*, 1990; Swegle *et al.*, 1992; Shih *et al.*, 2001). A special case among plant chitinases is represented by the enzyme from *Coix lachryma-jobi* seeds (the Job's tears plant, *Poaceae*), which also shows α-amylase inhibitory activity (Ary *et al.*, 1989). Its native structure, unique among plant chitinases, corresponds to a dimeric protein with two similar or identical subunits of about 26 kDa linked by disulphide bonds.

A list of cloned seed chitinase genes (complete sequences) is presented in Table 1. Of these, the two rye enzymes (*Secale cereale*, classes I and II) have also been completely sequenced at the protein level (Yamagami and Funatsu, 1993b, 1994). No significant differences are found among the different members of a given class. The only exception is the putative class III enzyme from soybean seeds that has a C-terminal extension of 31 residues and for which a new class, Chib2, has been proposed (Yeboah *et al.*, 1998). The rice (*Oryza sativa*) enzyme is the only class VII member described so far in seeds. It was identified as a highly abundant husk protein that might be related to dormancy (Nakazaki *et al.*, 1997).

To analyse the genetic relationships of seed chitinases, we have constructed a phylogenetic tree using the complete sequences of all class I, II, IV and VII enzymes shown in Table 1. Class III enzymes were excluded from the comparison because they differ significantly from the other chitinases (Collinge *et al.*, 1993; Henrissat, 1999). The topology of the resulting phylogram (Fig. 2) allows the following conclusions to be drawn: (1) class IV and VII enzymes (which include the rice chitinase and those members involved in somatic embryogenesis) constitute a separate group; the presence of angiosperm and gymnosperm enzymes in this group suggests that its divergence took place before the branching out of both taxa (see Araki and Torikata, 1995; Roger *et al.*, 1998); and (2) the separation between class I and II enzymes must have occurred after monocots and dicots diverged. Essentially the same results are obtained when only catalytic domains are considered (not shown). Standard bootstrap analysis further strengthens these conclusions.

In the few cases analysed, class I and III chitinases appear to be encoded by single-copy genes or small families of several genes (Leah *et al.*, 1991; Wu *et al.*, 1994; Yeboah *et al.*, 1998; Gijzen *et al.*, 2001). The

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Table 1. List of cloned seed chitinases (only complete sequences). The sequence of the class I enzyme from *Hordeum vulgare* has been obtained from a genomic clone. The EMBL Nucleotide Sequence Database accession numbers are indicated

Organism	Class	Accession no.	Reference
Castanea sativa	I	X95610	Allona *et al*. (1996)
Glycine max	I	AF335589	Gijzen *et al*. (2001)
Hordeum vulgare	I	U02287	Ignatius *et al*. (1994)
Secale cereale	I	AB051578	Unpublished
Zea mays	I	L00973	Wu *et al*. (1994)
Canavalia ensiformis	II	AJ006992	Hahn *et al*. (2000)
Cucumis melo	II	AF241267	Unpublished
Hordeum vulgare	II	L34210	Leah *et al*. (1991)
Secale cereale	II	AB051579	Ohnuma *et al*. (2002)
Benincasa hispida	III	AF184884	Shih *et al*. (2001)
Cucumis melo	III	AF241266	Unpublished
Glycine max	III	AB000097	Yeboah *et al*. (1998)
Sphenostylis stenocarpa	III	AF137070	Colucci *et al*. (1999)
Daucus carota	IV	U52845	Kragh *et al*. (1996)
Picea glauca	IV	L42467	Dong and Dunstan (1997)
Zea mays	IV	M84164	Huynh *et al*. (1992)
Zea mays	IV	M84165	Huynh *et al*. (1992)
Oryza sativa	VII	AB054687	Nakazaki *et al.* (1997)

regulation of a class II chitinase gene, specifically expressed in barley aleurone, has been analysed through promoter deletions. A region was identified (–200 to –140) containing *cis*-acting sequences responsible for gene activation in aleurone, but silencing in leaves (Leah *et al*., 1994).

The subcellular location of seed chitinases, identified either through immunoelectron microscopy or by cell fractionation techniques, has not been investigated as yet. All the enzymes listed in Table 1 lack the C-terminal extension responsible for vacuolar targeting (Neuhaus *et al*., 1991, 1994), which suggests that they are secreted to the extracellular matrix. In agreement with this hypothesis, secretion of seed chitinases during imbibition (from the aleurone and scutellum) or in cell suspension cultures has been reported (Kragh *et al*., 1991; de Jong *et al*., 1992; Swegle *et al*., 1992; Jensen, 1994; Seetharaman *et al*., 1996).

Structure and catalytic mechanism

Most, if not all, glycosyl hydrolases are thought to act by general acid catalysis involving carboxylic residues. Such acid-catalysed hydrolysis can occur through one of two mechanisms, leading to either retention or inversion of the anomeric configuration at the newly formed reducing end (Sinnot, 1990). On the basis of their primary structure, all known seed chitinases can be grouped into two families of glycosyl hydrolases, 18 and 19 (Henrissat and Bairoch, 1993). Class I, II, IV and VII enzymes have homologous catalytic domains and are included in family 19, whereas class III chitinases belong to the more divergent family 18 (see Fig. 1). Although members of both families catalyse the random cleavage of internal β(1→4) glycosidic linkages in the chitin polymer and related C2 *N*-acetylated substrates, they differ substantially in structure and catalytic mechanism (Robertus and Monzingo, 1999). While family 19 enzymes have an ancient core structure of α-helices and display an inverting mechanism (single displacement), family 18 chitinases are essentially $(\alpha\beta)_8$-barrels that operate by a retaining mechanism (double displacement).

Family 19 chitinases

There are only two members of this family for which an X-ray structure has been solved, the barley chitinase Horv2 (PDB entries 2baa and 1cns; Hart *et al*., 1993, 1995; Song and Suh, 1996) and a jack bean chitinase (PDB entry 1dxj; Hahn *et al*., 2000). Both enzymes have been isolated from seeds, where chitinases are known to accumulate abundantly. Horv2 and the jack bean chitinase are monomeric class II enzymes, i.e. they lack the chitin-binding domain present in other members of family 19 (Fig. 1). Both proteins have very similar topologies, and superposition of their models (solved at 1.8 Å) yields a root mean square (rms) deviation of only 1.0 Å for all C^{α} positions. The overall fold corresponds to a compact α-helical domain with three conserved disulphide bridges. Ten helices and a small three-stranded β-sheet encompass about one-half of the linear sequence, with the remaining residues essentially in

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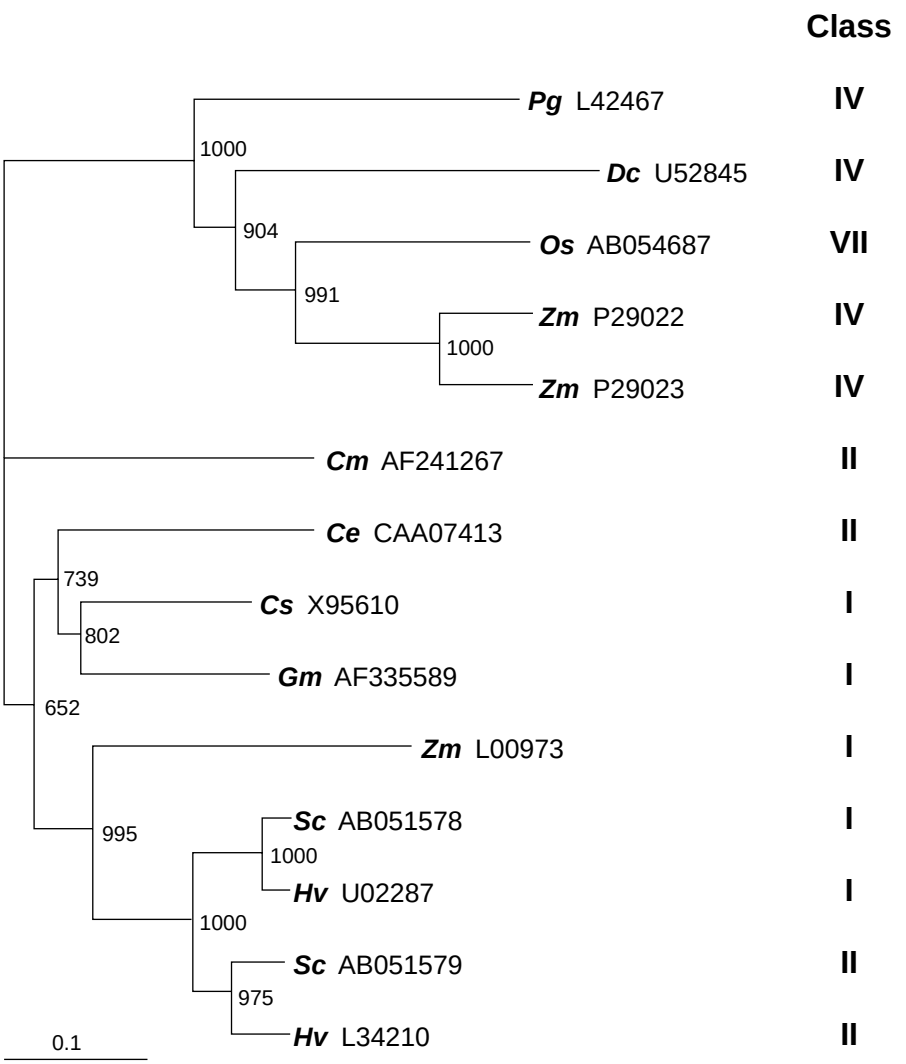

Figure 2. Phylogram of seed chitinases (classes I, II, IV and VII). The phylogenetic tree was constructed using the neighbour-joining method of CLUSTAL W (Thompson *et al.*, 1994). For each protein the accession number and a species identifier are listed: *Ce, Canavalia ensiformis*; *Cm, Cucumis melo*; *Cs, Castanea sativa*; *Dc, Daucus carota*; *Gm, Glycine max*; *Hv, Hordeum vulgare*; *Os, Oryza sativa*; *Pg, Picea glauca*; *Sc, Secale cereale*; *Zm, Zea mays*. The figure at each branching point shows the number of bootstrap replicas (out of 1000) giving rise to the depicted topology. Horizontal branch length reflects the distance between different chitinases.

loops connecting these structural elements. An elongated groove that runs along one side of the protein is presumed to be responsible for substrate binding and catalysis.

The finding that family 19 chitinases and lysozymes are structurally similar (Hölm and Sander, 1994) facilitated the modelling of hexasaccharide model substrates into the hypothetical active site of Horv2 (Hart *et al.*, 1995; Brameld and Goddard, 1998). This provided a view of likely substrate interactions and led to the hypothesis that two acidic residues, Glu67 and Glu89, are directly involved in the catalytic mechanism (Hart *et al.*, 1995; Song and Suh, 1996). Previously, a tyrosine residue had been recognized as relevant for productive substrate binding, but not for catalysis (Verburg *et al.*, 1992, 1993). Using knowledge-based protein modelling, as well as structural and sequence comparisons, Garcia-Casado *et al*. (1998) identified Glu124 and Glu146 as the potential catalytic residues of a highly abundant class I chitinase in chestnut seeds. The involvement of the proposed glutamate residues in catalysis has been confirmed for the barley and chestnut enzymes by site-directed mutagenesis (Andersen *et al.*, 1997; Garcia-Casado *et al.*, 1998). These authors also mutated other conserved active-site residues and

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analysed the effects of such replacements on chitinolytic activity. A similar study has also been conducted for a family 19 enzyme from tobacco leaves (Iseli-Gamboni *et al*., 1998).

Stereochemical studies have shown that family 19 enzymes hydrolyse their substrate with overall inversion of the anomeric configuration (Fukamizo *et al*., 1995; Iseli *et al*., 1996; Hollis *et al*., 1997). In this mechanism, one glutamate (Glu67 in Horv2) acts as a general acid that attacks the glycosidic bond, and the other glutamate (Glu89 in Horv2) acts as a general base that activates a water molecule on the other side of the bond. In the chestnut enzyme, the O^{γ} atom of Thr175 seems a likely candidate to hydrogen bond to such a water molecule, an assumption supported by the fact that the non-conservative substitution Thr175→Ala caused a significant decrease in specific activity (Garcia-Casado *et al*., 1998).

Family 18 chitinases

To date no seed chitinases belonging to this family have been crystallized. However, the three-dimensional structures of several family 18 chitinases have been solved, including the plant class III enzyme, hevamine (PDB entry 2hvm; van Scheltinga *et al*., 1996); a bacterial chitinase from *Serratia marcescens* (PDB entry 1edq; Perrakis *et al*., 1994; Papanikolau *et al*., 2001); and a cloned chitinase from the pathogenic fungus *Coccidioides immitis* (PDB entry 1d2k; Hollis *et al*., 2000). Since all plant chitinases of class III have very similar sequences, it seems likely that the seed members (see Table 1) will have the same overall fold as hevamine. Indeed, all the crystallized enzymes of this family have a common tertiary structure in spite of the low sequence similarity between plant and bacterial enzymes. The enzyme core corresponds to an $(\alpha\beta)_8$-barrel, with eight parallel strands of sheet and eight return helices forming a ring towards the outside. The substrate-binding cleft is located at the carboxy-terminal end of the β-barrel. A similar architecture has also been found in other polysaccharide-hydrolysing enzymes, such as α- and β-amylases, cellobiohydrolases and β-glucanases (van Scheltinga *et al*., 1996).

Conversely to family 19 chitinases, the enzymes of family 18 operate by a retaining mechanism (Armand *et al*., 1994; Fukamizo *et al*., 1995; Iseli *et al*., 1996). The mechanism of hen egg-white lysozyme, a widely held paradigm for retaining β-glycosyl hydrolases, involves a catalytic acid/base carboxylate residue (Glu35), which protonates the glycosidic oxygen, and a deprotonated carboxylate (Asp52). Although the latter residue has long been thought to stabilize an oxocarbenium ion intermediate (e.g. Strynadka and James, 1996), recent evidence supports the alternative idea that Asp52 is involved in forming a covalent glycosyl–enzyme intermediate in lysozyme (Vocadlo *et al*., 2001), as previously postulated by Koshland (1953). Based on model-building studies, the family 18 chitinases were also thought initially to act via a long-lived oxocarbenium intermediate (Perrakis *et al*., 1994). However, detailed structural analyses of enzyme complexes with allosamidine (a substrate analogue) and oligosaccharide substrates led to the proposal of substrate-assisted catalysis, with a glutamic acid acting as the single catalytic residue (van Scheltinga *et al*., 1995, 1996; Tews *et al*., 1996, 1997). Variations of this mechanism have recently been put forward for chitinase A from *S. marcescens* (Papanikolau *et al*., 2001) and hevamine (Bortone *et al*., 2002). Interestingly, an active-site aspartate residue has been identified in both instances, which is relevant for catalysis, although with distinct putative roles. In hevamine, Asp169 was proposed to ion pair with the transition state, while the equivalent residue of chitinase A (Asp313) was proposed to help position the catalytic glutamate. In the same regard, site-directed mutagenesis of the family 18 chitinase from *Bacillus circulans* has identified both Asp200 and Glu204 as essential residues for catalysis (Watanabe *et al*., 1993). Clearly, more experimental data will be needed to determine whether or not family 18 chitinases use a 'Koshland' mechanism.

Antifungal activity

In general, a plant molecule is considered as part of a defensive mechanism against pathogenic microorganisms when: (1) its synthesis is induced in response to pathogen challenge; (2) its expression level is dependent on specific race–cultivar interactions; and/or (3) it shows antimicrobial activity either *in vivo* or, more often, in fungal or bacterial growth inhibition assays (Kombrink and Somssich, 1995). There is wide agreement that plant chitinases from vegetative tissues are involved in defence against pathogens, either directly through their antifungal properties or indirectly through the release of chitin oligomers capable of eliciting plant defensive responses. Furthermore, chitinases and other defensive proteins, such as glucanases, osmotins, thionins, lipid transfer proteins or α-amylase and proteinase inhibitors, accumulate abundantly in seeds as part of their normal developmental programme (Bol *et al*., 1990; Garcia-Olmedo *et al*., 1992; Broekaert *et al*., 1997; Yun *et al*., 1997). Such abundance and multiplicity of defensive proteins in seeds is probably explained by the fact that their storage tissues are good substrates for a wide range of heterotrophic organisms. These tissues must be particularly sensitive to microbial attack when their water content is highest, i.e. during seed development and

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germination. In agreement with this notion, the highest accumulation of chitinase mRNA has been consistently observed during mid- and late maturation stages in the seeds of different plant species (Leah *et al*., 1991; Huynh *et al*., 1992; Swegle *et al*., 1992; Garcia-Casado *et al*., 2000). The induction of seed chitinases by fungal attack has been demonstrated in some monocot species during both maturation and germination. For example, two genes encoding class I chitinases are induced in developing maize kernels after inoculation with *Aspergillus flavus*. These genes were activated in the embryo and the aleurone layer, but not in the endosperm (Wu *et al*., 1994). More recently, Ji *et al*. (2000) have shown that *A. flavus* and *Fusarium moniliforme* induce the expression of different chitinase isoforms, suggesting some level of specificity in the response. During germination, the latter fungus also triggered the induction of three chitinases and a β-1,3-glucanase in maize embryos. When seedlings were later analysed, the accumulation patterns of glucanase and chitinase isoforms in radicles and coleoptiles appeared to be distinct from those seen in embryos (Cordero *et al*., 1994). The induction of different chitinase isoforms and other PR proteins has also been documented in germinating wheat seeds inoculated with *Fusarium culmorum* (Caruso *et al*., 1999). Much less attention has been devoted to the problem of specific race–cultivar interactions in the response of seed tissues to pathogen infection. In this context, analysis of different resistant and susceptible sorghum lines has shown a correlation between grain mould incidence and the levels of chitinase and other antifungal proteins (Rodriguez-Herrera *et al*., 1999; Bueso *et al*., 2000).

The antifungal properties of a number of purified seed chitinases, in particular from cereal species, have been investigated extensively. Fungal pathogens relevant in agriculture and non-pathogenic model species have been used to test the inhibitory effects of purified class I, II and IV enzymes from monocots (wheat, barley, sorghum and maize) and dicots (chestnut) (Roberts and Selitrennikoff, 1988; Collada *et al*., 1992; Huynh *et al*., 1992; Swegle *et al*., 1992; Allona *et al*., 1996; Garcia-Casado *et al*., 1998; Krishnaveni *et al*., 1999; Taira *et al*., 2001). These studies used microtitre well plate assays or disc-agar plate assays to monitor hyphal growth or fungal spore germination. In the first case, the amount of purified protein needed to inhibit 50% of hyphal growth (IC_{50}) varied between 0.1 and 2 μM. When the second assay was used, growth inhibition was observed with as little as 0.5 μg of protein per disc. Some of the purified enzymes were effective against phytopathogenic fungi such as *Fusarium sporotrichoides*, *F. oxysporum*, *F. moniliforme*, *Alternaria solani* or *Rhizoctonia solani*, but not against *Sclerotinia sclerotiorum* or *Gaeumannomyces graminis*. No inhibition has been observed when pathogenic oomycetes such as *Phytophtora infestans* or *Pythium myrtotylum*, which do not contain chitin in their cell walls, were assayed (Huynh *et al*., 1992; Swegle *et al*., 1992). Synergistic antifungal effects have been reported for combinations of chitinases and other seed defensive proteins. Hejgaard *et al*. (1991) and Leah *et al*. (1991) showed that mixtures of a barley seed chitinase with a β-1,3-glucanase, two thaumatin-like proteins, and a ribosome-inactivating protein from the same organ, had synergistic effects against *Trichoderma viride* and *F. sporotrichoides*. Analogous results have also been reported for a class I chitinase and a thaumatin-like protein purified from chestnut cotyledons (Garcia-Casado *et al*., 2000). The antifungal activity of seed chitinases has been confirmed *in planta* by Jach *et al*. (1995), who also showed synergistic effects with other defensive proteins. For this purpose, they obtained transgenic tobacco plants expressing a class II chitinase (CHI), a β-1,3-glucanase (GLU) and a ribosome-inactivating protein (RIP) from barley seeds, as well as plants transformed with tandemly arranged CHI and GLU or CHI and RIP constructs. The latter plants performed significantly better when infected with *R. solani* than those expressing a single barley protein at similar levels.

Despite the abundant literature on the antifungal properties of plant chitinases and their possible role in plant defence, their mode of action is not yet understood. Both chitin synthesis inhibition due to the breakdown of nascent chains and the release of chitin fragments from isolated cell walls have been demonstrated *in vitro* using purified enzymes (Molano *et al*., 1979; Boller *et al*., 1983; Mauch *et al*., 1988; Brunner *et al*., 1998). Moreover, chitin breakdown has been observed when a bean chitinase was applied to actively growing mycelial cells of *R. solani* (Benhamou *et al*., 1993). Antifungal activity has been reported for chitinases that contain an N-terminal chitin-binding domain (hevein domain; classes I and IV) and also for enzymes that lack such a domain. At the same time, mature hevein, stinging nettle agglutinin (UDA; built of two hevein-like domains), and some structurally related non-enzymatic peptides from amaranth also inhibit fungal growth (Broekaert *et al*., 1989, 1992; Chrispeels and Raikhel, 1991; van Parijs *et al*., 1991; Raikhel *et al*., 1993). To analyse the possible role of the hevein domain in antifungal activity, Iseli *et al*. (1993) expressed a wild-type tobacco class I chitinase and a truncated form lacking the hevein domain in transgenic *Nicotiana sylvestris* plants. By comparing both proteins, they concluded that the hevein domain is essential for chitin binding, but not for catalytic or antifungal activity. Results with seed chitinases support the idea that the chitin-binding domain alone can interfere with hyphal growth, as suggested by the

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above studies with non-enzymic lectins. By using site-directed mutagenesis, different forms of a class I chestnut chitinase (CsCh3) were generated in our laboratory, some of which had impaired chitinolytic activity. The effects of these variants, wild-type CsCh3, and the homologous class II enzyme, CsCh1, on the fungus *Trichoderma viride*, revealed that catalysis is not necessary for antifungal activity (Garcia-Casado *et al*., 1998). Moreover, the morphological changes in the hyphal tips caused by the wild-type enzyme and all its mutated forms were different from those caused by the class II enzyme. Thus, it appears that the two domains present in class I chitinases, the chitin-binding domain and the chitinolytic domain, can alter apical growth, although through different mechanisms (Garcia-Casado *et al*., 1998). Interestingly, in a similar study conducted with a class II enzyme from barley grains, those mutants that lacked chitinolytic activity still retained some ability to inhibit the growth of *T. viride*. By contrast, no inhibition was found when the heat-inactivated wild-type chitinase was assayed (Andersen *et al*., 1997). The residual antifungal activity of the inactive mutant forms might be due to their ability to bind chitin. In line with these results, recent analyses of a class I tobacco chitinase and informative recombinant forms have led to the suggestion that the hevein domain has intrinsic antifungal activity (Suarez *et al*., 2001). However, Taira *et al*. (2001) have suggested that the hevein domain of a rye grain chitinase lacks antifungal activity. Another relevant comparison was made between two purified class IV enzymes from maize grains, structurally highly similar (87% identity), but markedly different in their antifungal activity (about tenfold). The enzyme showing more pronounced antimicrobial effects was that with higher chitinolytic activity (threefold) and lower substrate-binding constant (tenfold) (Huynh *et al*., 1992).

Additional functions

It is likely that constitutive chitinases and other PR proteins are synthesized primarily to protect seeds and other non-vegetative organs from microbial attack. However, increasing evidence suggests that at least some seed chitinases may also be involved in non-defensive functions. For example, de Jong *et al*. (1992) demonstrated that a mutant cell line of carrot, unable to form embryos, was rescued by EP-3, a glycosylated class IV chitinase from wild-type carrot cells. The mutant phenotype did not result from structural differences in the enzyme, but from a transient decrease in its amount compared to the wild type (de Jong *et al*., 1995). Further studies revealed that there are at least four *EP3* genes in carrot and that the proteins encoded by two of them had different effects on embryo formation (Kragh *et al*., 1996). In addition, a class I chitinase able to rescue the mutant line was also identified in this work. *EP3* genes are expressed in the inner tegument of young carrot fruits and in a specific subset of endosperm cells, but not in zygotic embryos (van Hengel *et al*., 1998). The function of EP-3 in embryogenesis involves the modification of GlcNAc/GlcN-containing arabinogalactan proteins. Indeed, pretreatment of such proteins with EP-3 resulted in increased activity to restore embryogenesis in cell-culture-derived protoplasts (van Hengel *et al*., 2001). Interestingly, Nod-like factors might also be involved in this process, since several Nod factors have also been shown to rescue the mutant line (de Jong *et al*., 1993; Dénarié and Cullimore, 1993). Somatic embryogenesis-related chitinases have been described in other plant species such as *Picea abies, Picea glauca, Linum usitatissimum, Pinus caribaea* and a *Cichorium* hybrid (Dong and Dunstan, 1997; Egertsdotter and von Arnold, 1998; Roger *et al*., 1998; Domon *et al*., 2000; Helleboid *et al*., 2000). In *Pinus caribaea,* a 48 kDa chitinase-like protein secreted by embryogenic tissues acts on arabinogalactan proteins extracted from the same cells (Domon *et al*., 2000).

Certain chitinases and β-1,3-glucanases are specifically expressed in tomato endosperm prior to radicle emergence, and this has led to speculation for a possible role in seed germination (Wu *et al*., 2001). However, direct evidence that these enzymes participate in cell wall modification or tissue weakening is still lacking. In addition, abscisic acid (ABA) does not seem to affect chitinase expression during tomato seed germination (Wu *et al*., 2001).

Finally, some cold-inducible chitinases from winter rye leaves possess antifreeze activity (Hon *et al*., 1995; Hiilovaara-Teijo *et al*., 1999). Such activity could be important to protect seed tissues from frost damage in temperate and boreal regions. Indeed, chitinases and other PR proteins accumulate at unusually high levels in recalcitrant chestnut seeds, which have, at shedding, one of the highest water contents known (Collada *et al*., 1992; Allona *et al*., 1996; Garcia-Casado *et al*., 1998, 2000). However, transgenic *Arabidopsis* plants constitutively expressing the major class I chitinase from chestnut seeds are no more tolerant to freezing temperatures than the control plants (our unpublished results).

Allergenic properties

Allergic diseases provoked by contact with latex-derived products are becoming an increasing occupational and public health problem (see Salcedo *et al*., 2001 for a recent review). About 50% of latex-allergic patients also show hypersensitive reactions to certain plant fruits. This has led to the proposal of a

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'latex–fruit syndrome' (Blanco *et al*., 1994; Beezhold *et al*., 1996). Chestnut, banana, avocado and kiwi have emerged as the foods implicated in this syndrome, and recent research has identified the class I chitinases present in such foods as major panallergens (Diaz-Perales *et al*., 1998; Mikkola *et al*., 1998; Sowka *et al*., 1998; Sanchez-Monge *et al*., 1999). By contrast, class II enzymes from the same sources, with highly similar catalytic domains but lacking the N-terminal hevein-like domain, show no allergenic activity when tested *in vitro* and *in vivo* (Diaz-Perales *et al*., 1998; Blanco *et al*., 1999). Since mature hevein is also a major latex allergen (Alenius *et al*., 1995), the hypothesis has been put forward that the hevein-like domain of class I chitinases is a major determinant of their allergenic properties (Diaz-Perales *et al*., 1998; Mikkola *et al*., 1998; Salcedo *et al*., 2001).

Certain seeds that contain high levels of class I chitinases, such as those of cereals and soybean, have not been associated with the latex–fruit syndrome. However, a putative class I enzyme from wheat flour is recognized by sera (IgE) from latex allergic patients (Diaz-Perales *et al*., 1999). The explanation for this contradiction probably lies in the thermal treatments to which cereal and legume seeds are subjected before being eaten. In this regard, the heat-inactivation of allergenic chitinases from bean and avocado has been demonstrated recently (Sanchez-Monge *et al*., 2000). In fact, only raw foods (seeds and fruits) have been associated so far with the latex–fruit syndrome. Nevertheless, it is also possible that some class I chitinases are not reactive because their hevein-like domains show low sequence identity to latex hevein. Whereas such identity amounts to *c*. 70% in the case of chestnut, banana and avocado chitinases, it can be as low as 49% in other cases.

Future perspectives

While significant advances have been made regarding the three-dimensional structure and action of class I, II and III enzymes, much less is known of other chitinases. Some of them are abundantly expressed in seeds, and this should facilitate their isolation and structural characterization. Such studies may reveal further details on the action of chitinases (e.g. the effects of the deletions in the hevein-like and catalytic domains of class IV enzymes). More research is clearly necessary to determine whether the different isoforms present in a given tissue act synergistically for a common purpose or have different functions. Another aspect of interest is the fact that some chitinases may play roles in non-defensive functions. For example, Nod factors and seed arabinogalactan proteins are substrates for chitinases, which suggests involvement of these enzymes in plant morphogenesis, plant cell fate or plant–microbe interactions. These possibilities are supported by the specific activities shown by different chitinases towards substrates such as chitin, chitosan, Nod factors and peptidoglycans. In any case, there is little doubt that the possible involvement of plant chitinases in non-defensive functions will continue attracting the interest of researchers. Finally, the biotechnological potential of these enzymes warrants future efforts to elucidate their allergenic properties. The role of the hevein-like domain present in class I and IV enzymes must be clarified regarding latex–plant food co-sensitization. Likewise, more studies are needed on chitinases that lack this domain, but are involved in other types of allergies.

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