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Conifer Chitinases

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ABSTRACT

Over the last two decades scientists have focused much attention on the physiological, molecular and functional biology of plant chitinases and there is considerable evidence suggests that chitinases play important roles in plant defense systems. Chitinases have also been shown to play a role in plant growth and development. Several review articles exist for chitinases of angiosperms but there is no such review for conifer chitinases, despite the economic and ecological significance of coniferous species in the world's forests. Conifer chitinases consist of at least several classes of enzymes that are represented by small gene families. Class II (acidic) and class IV (basic) chitinases, expressed differentially over time and space, have been shown to be the major defense players in many conifer pathosystems. Class I and III chitinases are also reported in some conifers. This review discusses the current body of knowledge regarding conifer chitinases, including the molecular structure of chitinase genes and their regulation and function in conifer plants. Future potential uses for conifer chitinases as biopesticides and agents of biofuel production are also discussed.

Keywords: biocontrol, biofuel, chitin binding domain, growth and development, host defense, PR proteins
Abbreviations: **AFP**, antifreeze protein; **AGP**, arabinogalactan protein; **DF**, Douglas-fir; **ECP**, endochitinase-like protein; **EST**, expressed sequence tag; **LCO**, lipo-chitooligosaccharides; **PCD**, programmed cell death; **PEM**, pro-embryogenic masses

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INTRODUCTION

Conifer trees are the dominant species in both boreal and temperate forests around the globe, wherein they play vital roles in ecosystem functions (Bonello *et al.* 2006). Regeneration of conifer forests, either naturally or by planting high quality seedlings, is essential for maintaining the world's large forested areas as active carbon dioxide (CO_2) sinks after harvesting (Holopainen *et al.* 2009) and natural disturbances. The future composition of the world's forests and their sustainability will be greatly affected by both global warming and increased pressure by pests and pathogens, many of which are expected to expand in range and amplitude (Niemelä *et al.* 2001; Sturrock *et al.* 2006). To successfully manage the present and future pathogens affecting the health of conifer forests we must understand host-pathogen interactions at a molecular level, including knowledge of the regulation and function of major pathogenesis-related genes and proteins in conifer pathosystems.

Plant chitinases (EC 3.2.1.14) are of particular importance to fungal pathosystems because all true fungi contain chitin as a primary structural component of their cell walls (Wessels 1994). Chitin is a linear homopolymer of *N*-acetyl-D-glucosamine and it is hydrolyzed by chitinases into smaller oligomers or monomers (Bishop *et al.* 2002; Xiao *et al.* 2007; Xu *et al.* 2007; Nakamura *et al.* 2008). Many stu-

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Invited Review

dies suggest that conifer chitinases play their most significant roles in conifer defense against pathogens. In addition to that, some conifer chitinases are also induced by abiotic stresses.

During the past decade, chitinases in general have received increased attention because of their wide range of potential uses. Chito-oligomers produced by enzymatic hydrolysis have valuable applications in medicine, agriculture, and industry through their antibacterial, antifungal, hypocholesterolemic, and antihypertensive properties. They also have use as a food quality enhancer (Patil *et al.* 2000; Bhattacharya *et al.* 2007). Recent papers provide some details on the prospects chitinases have in biocontrol (Koga 2005; Quecine *et al.* 2008) and biofuel production (Vaaje-Kolstad *et al.* 2005; Himmel *et al.* 2007; Eijsink *et al.* 2008). Although there are many research papers available on plant chitinases, including several review articles that highlight chitinases of short-lived angiosperm crops (Flach *et al.* 1992; Collinge *et al.* 1993; Punja and Zhang 1993; Beintema 1994; Graham and Sticklen 1994; Meins *et al.* 1994; Araki and Torikata 1995; Iseli *et al.* 1996; Hamel *et al.* 1997; Selitrennikoff 2001; Bishop *et al.* 2002; Kasprzewska 2003; Lucca *et al.* 2005), there is no broad-scale report on conifer chitinases. To the best of our knowledge, this is the first review article on the current state of knowledge on conifer chitinases, including their regulation and function.

CLASSIFICATION AND STRUCTURAL DIVERSITY OF CONIFER CHITINASES

In most plants, including conifers, chitinases occur as diverse groups that differ in their primary structure, isoelectric point and cellular localization (Collinge *et al.* 1993; Beintema 1994; Graham and Sticklen 1994; Meins *et al.* 1994; Kasprzewska 2003; Hietala *et al.* 2004; Fossdal *et al.* 2006, 2007; Islam *et al.* 2010). In general, chitinases have been classified into two glycosyl hydrolase (E.C.3.2.1.14) families: family 18 and family 19 (Henrissat and Bairoch 1993). Family 18 chitinases consist of general types: a 'plant type' with endogenous activity that generates products of varying length, and a 'bacterial type' with exogenous activity releasing chitobiose or chitotriose from the non-reducing end of chitin (van Aalten *et al.* 2001; Andersen *et al.* 2005; Ubhayasekera *et al.* 2009). Family 18 chitinases hydrolyze the glycosidic bond with retention of the anomeric configuration, whereas family 19 chitinases have a different protein structure with an α-helical fold and hydrolyze with inversion. Family 18 chitinases occur in bacteria, fungi and some plants while family 19 glycosyl hydrolases occur mostly in plants. It is reported that chitinases within one family share similar three-dimensional structure and the same mechanism of hydrolytic action (Iseli *et al.* 1996).

The classification of plant chitinases has focused on the presence of auxiliary domains – namely, a chitin-binding domain, a hinge domain and a carboxy-terminal extension-flanking the main catalytic domain (Hamel *et al.* 1997). Plant chitinases are divided into seven classes (I-VII) based on their primary structures (Collinge *et al.* 1993; Meins *et al.* 1994; Neuhaus *et al.* 1996; Brunner *et al.* 1998; Gomez *et al.* 2002; Kasprzewska 2003). These seven classes fit into three of the 17 identified pathogenesis-related (PR) protein families (Neuhaus *et al.* 1996; Kasprzewska 2003). The family PR-3 includes chitinases of class I, II, IV, VI and VII; family PR-8 includes class III chitinases; and family PR-11 includes chitinases of class V (Neuhaus *et al.* 1996; Kasprzewska 2003). Additionally, some proteins of the family PR-4 with low endochitinase activity were found (Melchers *et al.* 1994; Neuhaus *et al.* 1996).

Based on previous reports (Collinge *et al.* 1993; Neuhaus *et al.* 1996; Hamel *et al.* 1997; Wiweger *et al.* 2003; Ubhayasekera *et al.* 2009), the schematic structural difference between classes of plant chitinases is presented in **Fig. 1**. Class I, II, IV and VII chitinases belong to the family 19 glycosyl hydrolase and share a high amino acid sequence identity within their catalytic domain. Furthermore, class I and IV chitinases have a cysteine-rich chitin-binding domain followed by a variable hinge region. The chitin-binding domain is absent in class II chitinases (Neuhaus *et al.* 1996; Ubhayasekera *et al.* 2009). Although classes IV and VII resemble classes I and II respectively, the former are significantly smaller due to some deletions. It is proposed that a basic class II chitinase is a putative ancestor of basic class I and acidic class II chitinase (Ohme-Takagi *et al.* 1998). Class IV chitinase genes, which are phylogenetically related to class I and II chitinase genes, are thought to have evolved from a class I chitinase gene by four deletions in the coding sequence (Araki and Torikata 1995). The protein genealogy of chitinases also suggests that class I and class II chitinase genes evolved from the same ancestral gene (Shinshi *et al.* 1990; Araki and Torikata 1995). In contrast, chitinases of class III, V and VI belong to the family 18 glycosyl hydrolase, are distributed in a wide range of organisms, including bacteria, fungi, plants, insects, mammals and viruses, and posses a common $(\alpha/\beta)_8$-barrel domain (Watanabe *et al.* 1999; Hollis *et al.* 2000).

To date, class I, II and IV chitinases have been reported from conifers (Davis *et al.* 2002; Karlsson *et al.* 2003; Hietala *et al.* 2004; Nagy *et al.* 2004; Karlsson 2005; Liu *et al.* 2005; Fossdal *et al.* 2006; Adomas *et al.* 2007; Fossdal *et al.* 2007; Adomas *et al.* 2008; Islam *et al.* 2010). However, the rapid progress in expressed sequence tags (ESTs) sequencing and molecular research is revealing additional classes of chitinases in conifer plants. For example, class III chitinase- (glycosyl hydrolase family 18) like ESTs were identified from several conifer species (Kusumi *et al.* 2002) and a class V chitinase (CrChii-A) was identified from *Cycas revoluta* Thunb. (Taira *et al.* 2009). Very recently the X-ray structure of the chitin-binding module and the catalytic module of a class IV chitinase has been reported from *Picea*

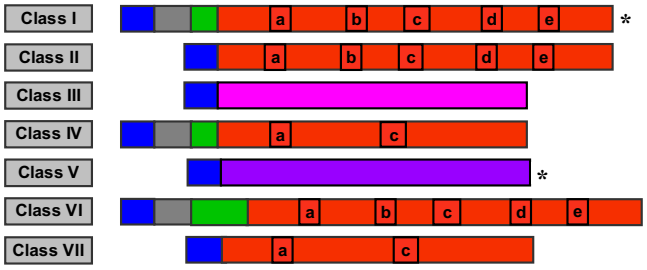


Fig. 1 Schematic representation of the structural differences between classes of plant chitinases. Signal peptides (blue), chitin binding modules (gray), hinge domains (green), and catalytic domains of chitinase family 19 (red) and two distinct groups of chitinase family 18 (pink and purple). Differences in the loop structures of catalytic domains of chitinase classes are indicated by the letters **a** (residues 164–170), **b** (217–222), **c** (235–257), **d** (308–311) and **e** (325–332) (adopted from Collinge *et al.* 1993; Hamel *et al.* 1997; Neuhaus *et al.* 1996; Wiweger *et al.* 2003; Ubhayasekera *et al.* 2009).

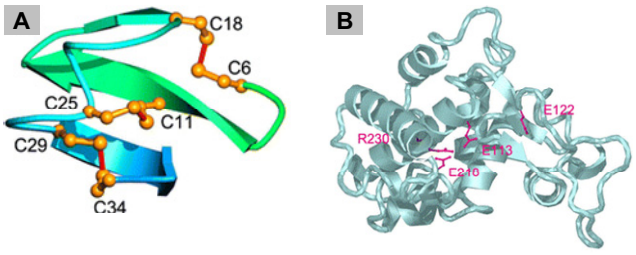


Fig. 2 Crystal structure of a basic class IV chitinase of *Picea abies*. (**A**) The chitin binding module (CtBM) of a class IV chitinase of *Picea abies* (color-coded from green to blue; disulfide-forming residues in gold). (**B**) General structure of the catalytic module (CM) of the same chitinase; residues that may be catalytically important are shown in pink (modified from Ubhayasekera *et al.* 2009). Letters show disulfide-forming cysteine residues (C), and catalytically active glutamic acid (E) and arginine (R) residues.

abies (L.) H. Karst. (**Fig. 2**; Ubhayasekera *et al.* 2009). This protein shows high homology with other conifer class IV chitinases (Liu *et al.* 2005; Islam *et al.* 2010). In addition, several PR-4 genes were identified from Douglas-fir (DF; *Pseudotsuga menziesii* (Mirb.) Franco) seedlings infected with *Phellinus sulphurascens* Pilát. These PR-4 genes were found to be upregulated significantly after *P. sulphurascens* infection. The nucleotide sequences of these genes showed very low identity with DF chitinase genes suggesting that DF PR-4 proteins may have a differential defense mechanism in infected DF plants (unpublished data). Further research is required to resolve this enigma.

PHYLOGENY OF CONIFER CHITINASES

The alignment of amino acid sequences for selected classes of chitinases from gymnosperms and angiosperms displays the sequence variability occurring within and between different chitinase classes (**Fig. 3**). In conifers, sequence similarity within class I is over 90%, whereas class II shows 37 to 98% and class IV shows 66 to 98% homology. Between conifer classes sequence identity is comparatively lower, ranging from 31 to 59%. When conifer peptide sequences were compared with relatively distant angiosperm species, the sequence identity was even lower, ranging from 3 to 68%. Based on the alignment data, a phylogenetic tree was constructed using MEGA version 4.0 (Tamura *et al.* 2007). Class V and VI were not included in this study because they are not very common *in planta* and to our best knowledge there is no record available for these classes in conifer species. The phylogenetic analysis revealed the presence of several distinct clades for gymnosperm and angiosperm classes. Conifer chitinases form two major groups. One group consists of class II and class IV chitinases; this group shares high homology with angiosperm class IV chitinases but forms a sub-group in the tree. The second group consists of class I and II conifer chitinases along with angiosperm class I, II and VII chitinases. Angiosperm class III chitinases were found to be very distinct in the tree (**Fig. 4**).

Although conifer chitinases of class I, II and IV show significant sequence similarities, like angiosperm chitinases they differ in several ways: 1) the chitin-binding domain of class IV has cysteines at seven of eight positions in common with class I chitinases, but the remainder of their sequences vary greatly, while class II lacks this domain; 2) there are four deletions in class IV chitinases, one within the chitin-binding domain and three within the catalytic domain, shortening the final protein product compared to class I chitinases; and 3) a C-terminal extension found in most class I chitinases is missing in both class II and IV chitinases (Graham and Sticklen 1994). It is reported that both class II and IV chitinases are secreted to the apoplast (Graham and Sticklen 1994; Singh *et al.* 2007) and these two classes show high homology in their catalytic domain; however, they do not show any significant homology with class III and V chitinases (Beintema 1994; Kasprzewska 2003).

SUBCELLULAR LOCALIZATION OF CONIFER CHITINASES

To better understand the functions of chitinases in plants, investigators have used different techniques to study their localization. In angiosperms, for example, subcellular localization of a tomato (*Lycopersicon esculentum* Mill.) chitinase (molecular mass of 26 kD) was studied using tomato root tissues infected with *Fusarium oxysporum* Schltdl. The enzyme was found to accumulate in areas where host cell walls were in close contact with fungal cells. In contrast, the enzyme could not be detected in vacuoles and intracellular spaces (Benhamou *et al.* 1990). It was also reported that beet necrotic yellow vein virus, the causal agent of rhizomania, induced accumulation of a chitinase protein in cell walls and extracellular spaces in sugar beet (Burketová *et al.* 2003), while a basic class IV chitinase was found to be localized in the extracellular space of cucumber (Boller and Metraux 1988). Asiegbu *et al.* (1995) reported the occurrence and accumulation of chitinase in seedling roots of *P. abies* following challenge by the root-rot pathogen *Heterobasidion annosum* (Fr.) Bref. Using transmission electron microscope (TEM) and immunogold labelling techniques, they demonstrated that the enzyme was localized in protein aggregates in host tissues and in the cell walls of intercellular hyphae. The labelling intensity increased with infection time. The gold labelling was always higher in infected roots than in non-infected seedling roots. A similar labelling pattern was observed when this experiment was repeated using root samples inoculated with the saprophyte *Phlebiopsis gigantea* (Fr.) Jülich.

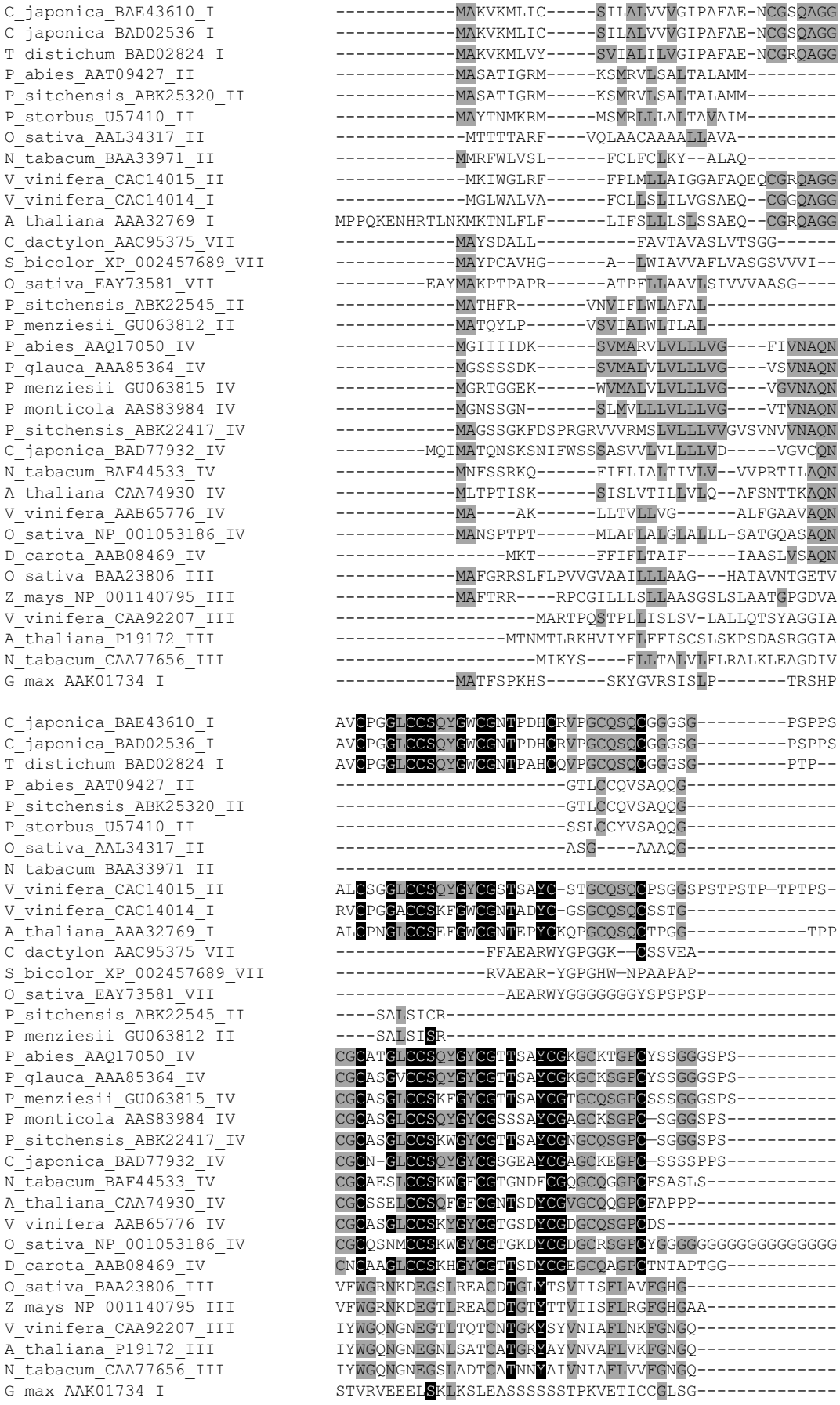
Similarly, an endochitinase-like protein (ECP) was found to be localized in the apoplastic fluid of DF needles (Zamani *et al.* 2003). Immunolocalization studies further suggested that this ECP protein is specifically localized in host cell membranes of DF seedlings (Islam *et al.* 2009). Infected host cell membranes frequently formed papillae where ECP were intensely localized. However, there was little or no localization of ECP observed in host cell walls, intercellular spaces, and cytoplasm of infected DF root tissues (**Fig. 5**; Islam *et al.* 2009). It is also reported that DF seedlings and mature trees contain multiple ECP isoforms (Zamani *et al.* 2003; Islam *et al.* 2009).

The localization of a class II chitinase in pine trees was successfully studied using suspension cell cultures of *Pinus elliottii* Engelm. (slash pine) and *Pinus taeda* L. (loblolly pine). Immunoreactive proteins were identified in the medium of both slash and loblolly pine cultures. In addition to that, one or more immunoreactive proteins approximately 32 kDa in size were detected in loblolly pine whole-cell extracts, suggesting the presence of other chitinase homologs that are localized in the vacuole (Davis *et al.* 2002). Based on the presence of a putative C-terminal vacuolar-sorting determinant (LIKTVV), this protein was designated as a class I chitinase as recommended by Neuhaus and Rogers (1998).

Collinge *et al.* (1993) and Neuhaus *et al.* (1996) have demonstrated that a short C-terminal extension of about six amino acids present in a tobacco basic class I chitinase is necessary for vacuolar localization. So far, vacuolar chitinases have been reported from several conifer species such as *P. abies* (Salzer *et al.* 1997a, 1997b; Hietala *et al.* 2004), *Pinus halepensis* Mill. and *P. taeda* (Davis *et al.* 2002; Sathyan 2004) and *Cryptomeria japonica* (L. f.) D. Don (Kusumi *et al.* 2002; Kado *et al.* 2003). Except for class I chitinases, no other conifer chitinases were recorded as vacuolar proteins. However, a class IV chitinase was recently cloned from yam that contains an additional sequence composed of eight amino acids at the C-terminal, when compared with class IV chitinases from other plants (Mitsunaga *et al.* 2004). In order to clarify the role of this C-terminal extension in cellular localization, Mitsunaga *et al.* (2004) conducted further studies using plants and suspension-cultured cells of *Nicotiana tabacum* L. These cultures, which were transformed with either the cloned yam class IV chitinase gene carrying the C-terminal extension or its truncated gene using the *Agrobacterium*-mediated method, suggest that the C-terminal extension of class IV chitinase plays an essential role as a targeting signal for plant vacuoles (Mitsunaga *et al.* 2004).

REGULATION OF CONIFER CHITINASES

Conifer plants exhibit both local and systemic expression of chitinases, regulated constitutively or induced by chemicals and pathogen infections. These systemic or inducible defense systems have evolved to deter or kill insects and inhibit or exclude pathogens physically and/or chemically (Bonello *et al.* 2006). Recent fossil evidence suggests that the defense mechanisms in conifers have been operating for at least the past 45 million years in the Pinaceae (Labandeira *et al.* 2001; Bonello *et al.* 2006).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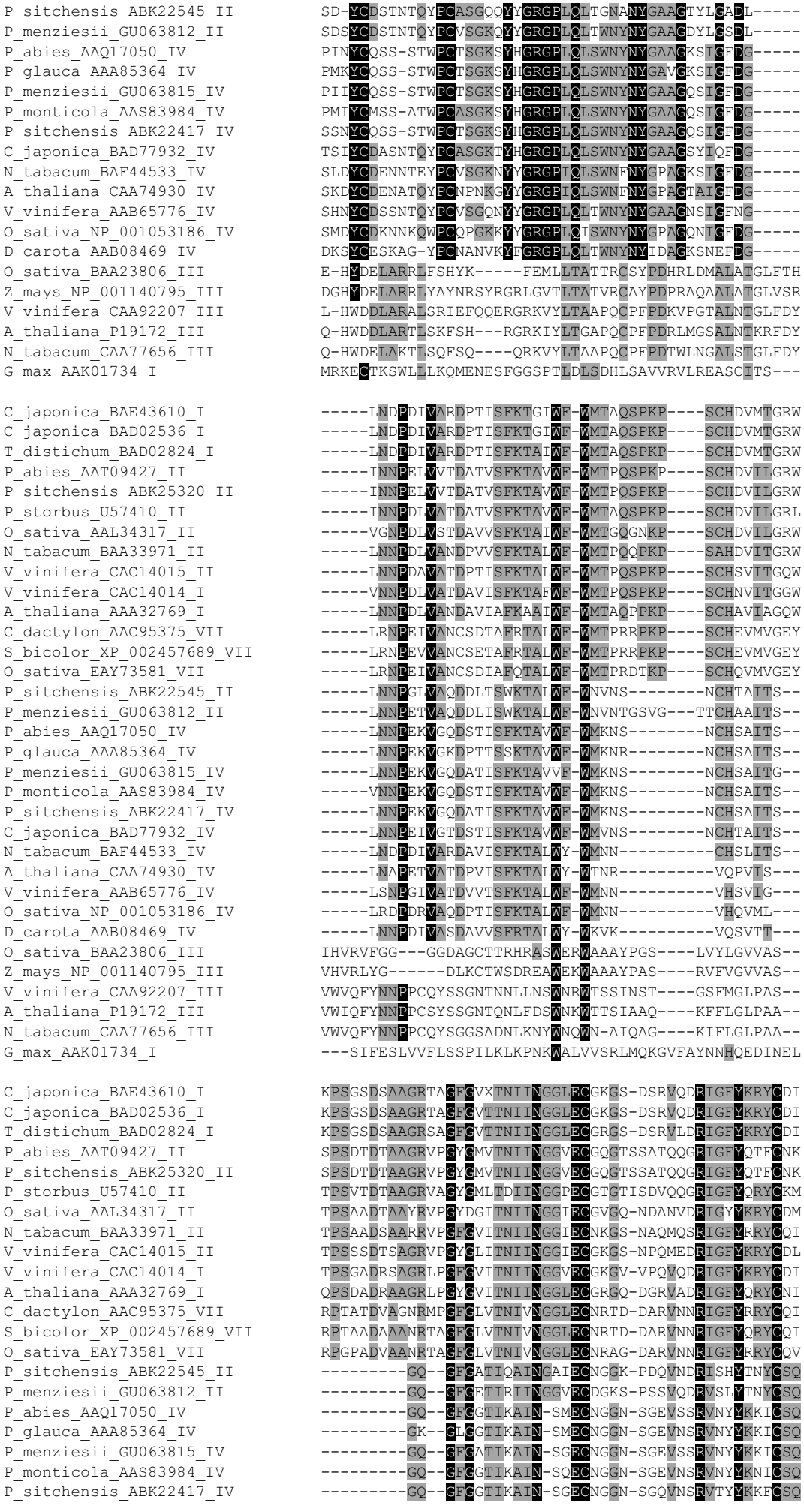
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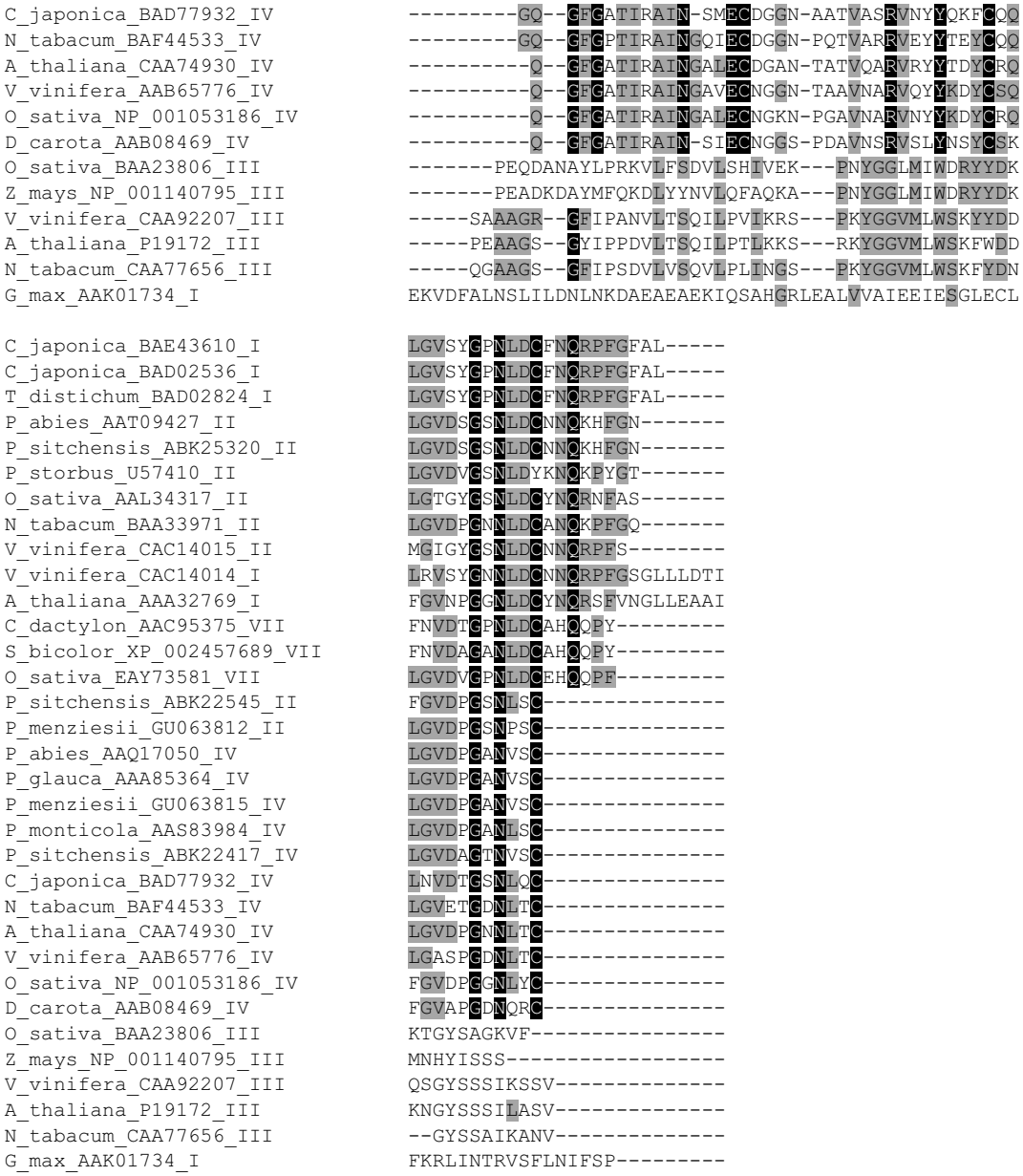

Fig. 3 Alignment of amino acid sequences for selected conifers and angiosperms chitinases. Each of the clones' ID include species name, NCBI accession number and the designated class number as I, II, III, IV and VII. Hyphens show gaps in sequences for the best alignment. Letters with black and gray background indicate amino acid residues that are identical in a wide (black) and a restricted (grey) range of chitinase classes and plant species, respectively. Plant chitinases are selected from the following species *Arabidopsis thaliana*, *Cryptomeria japonica*, *Cynodon dactylon*, *Daucus carota*, *Glycine max*, *Nicotiana tabacum*, *Oryza sativa*, *Picea abies*, *Picea glauca*, *Picea sitchensis*, *Pinus monticola*, *Pinus storbus*, *Pseudotsuga menziesii*, *Sorghum bicolor*, *Taxodium distichum*, *Vitis vinifera* and *Zea mays*.

Constitutive regulation of conifer chitinases

In healthy plants, some forms of chitinases, both vacuolar and apoplastic, are synthesized constitutively. Constitutive defense consists of a suite of stable defense products of varying properties that provide a generalized capacity for resistance to a broad range of organisms (Franceschi *et al.* 2005). Each of the constitutive defenses is determined by genetics and by the prior history of an organism. Constitutive regulation is also the first line of defense of all organisms, including plants, and it comprises a number of physical and chemical barriers (Bonello *et al.* 2006). Although morphological, anatomical and chemical structures are the major components of the constitutive defense of conifers, there are many defense-related proteins including chitinases that are constitutively regulated. Constitutive expression of chitinases is evident in many conifers including *P. menziesii* (Robinson *et al.* 2000; Zamani *et al.* 2003; Sturrock *et al.* 2007; Islam *et al.* 2009), *Pinus monticola* Doug. ex D. Don (Liu *et al.* 2005), *P. abies* (Wiweger *et al.* 2003; Hietala *et al.* 2004; Fossdal *et al.* 2006, 2007), *Picea glauca* (Moen.) Voss (Dong and Dunstan 1997), *Pinus sylvestris* L. (Pirttilä *et al.* 2002), *Taxus baccata* L. (Uzal *et al.* 2009), and *C. japonica* (Kusimi *et al*. 2002; Fujimura *et al.* 2005, 2007).

In *P. abies* a high level of constitutive chitinase expression has been reported. Differential regulation of a chitinase (PaChi1) was also observed after wounding and pathogen inoculation in the same plant. These data suggest that some chitinases may not have a major defense role, but may play an important role in plant growth and development (Hietala *et al.* 2004). Constitutively expressed chitinases may regulate plant development by generating signal molecules from endogenous substrates such as arabinogalactan proteins containing *N*-acetylglucosamine (Collinge *et al*. 1993; van Hengel *et al*. 2001). Specifically, constitutively expressed conifer chitinases may play a major role in somatic embryogenesis and seed development (Wiweger *et al.* 2003; dos Santos *et al.* 2006, 2008).

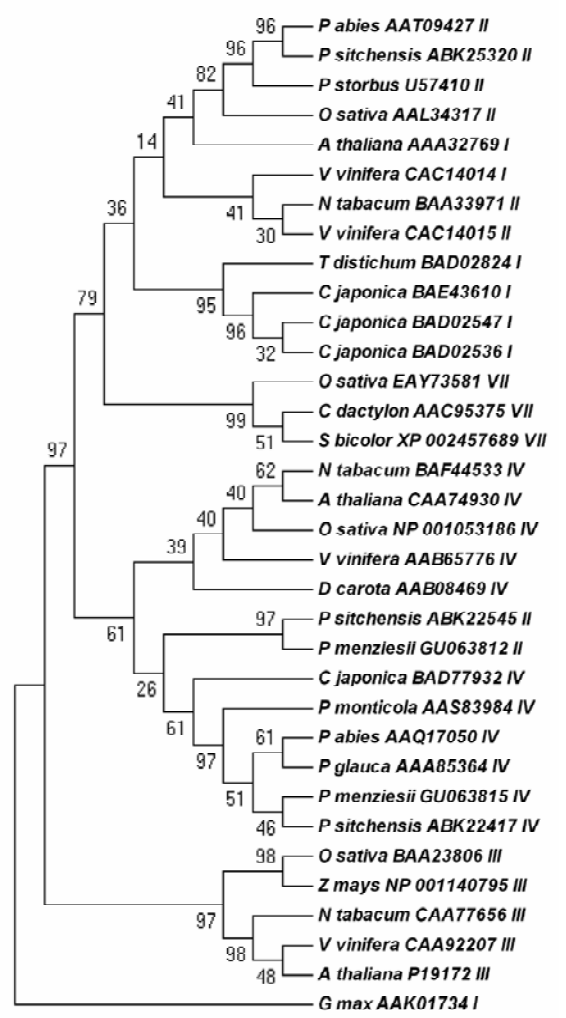

Fig. 4 Phylogenetic tree constructed using amino acid sequences of different classes of chitinases from gymnosperms and angiosperms. Using MEGA 4.0, construction of this tree was based on the full length coding regions of the amino acid sequences. Each of the clones' ID includes species name, NCBI accession number and the designated class number as I, II, III, IV and VII.

Inducible regulation of conifer chitinases

Inducible defense in planta is achieved through different PR proteins, signal chemicals and elicitors that respond under stress conditions to degrade components of invasive organisms, toxic proteins like porins and lectins, and inhibitors of enzymes such as proteinase and amylase inhibitors. PR protein-based inducible defenses can be highly specific to a particular organism (Franceschi *et al.* 2005). For instance, in *P. abies*, chitinases exist as a fairly large family of proteins, but only a small subset of this group may be upregulated during infection of a specific fungal pathogen (Hietala *et al.* 2004; Nagy *et al.* 2004).

The plant chitinase family appears to be a prominent component of the inducible defense of conifer species. Biotic and abiotic stress factors such as pathogens and pests (Hietala *et al.* 2004; Nagy *et al.* 2004; Liu *et al.* 2005; Sturrock *et al.* 2007; Islam *et al*. 2010), wounding (Fossdal *et al*. 2006; Ralph *et al.* 2006; Lippert *et al.* 2007), drought (Sathyan 2004; Lorenz *et al.* 2006) overwintering (Zamani *et al*. 2003; Jarząbek *et al.* 2009), elicitors (Wu *et al.* 1997; Mason and Davis 1997), inhibitors (Liu *et al.* 2005), and exogenous applications of signal chemicals (Kozlowski and Métraux 1998; Davis *et al.* 2002; Liu *et al.* 2005) may induce diverse chitinase isoforms in conifers.

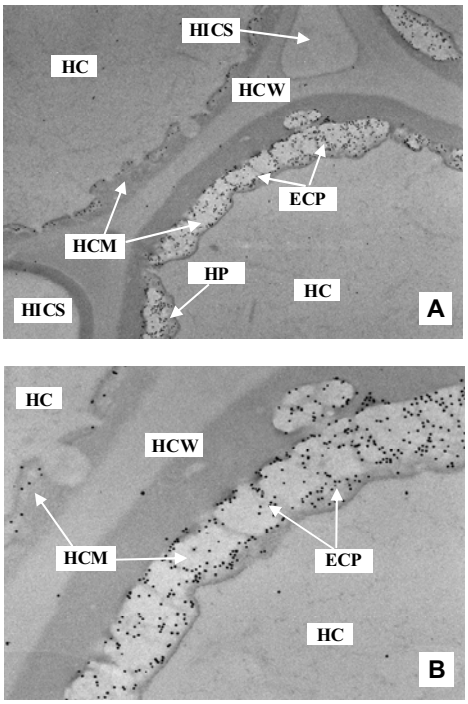

Fig. 5 Immunolocalization of a Douglas-fir endochitinase-like protein (ECP). (**A**) Transmission electron micrograph showing the localization of ECP in *Phellinus sulphurascens*-infected DF root tissues at 3200X magnification. (**B**) Higher magnification (5500X) of (**A**), showing distribution pattern of immuno-gold labelled ECP. HC – host cell, HCM – host cell membrane, HCW – host cell wall, HICS – host intercellular space, HP – host papillae, ECP – endochitinase protein, numerical legends show magnification for each micrograph (modified from Islam *et al.* 2009).

BIOLOGICAL FUNCTIONS

Available data indicate that conifer chitinases play important roles in plant defense, growth and development, and other physiological processes. Several investigators have reported on the biological functions and physiological processes of conifer chitinases; these are documented in **Table 1**. Important functional categories of conifer chitinases are presented below.

Conifer chitinases in defense against pathogens

Many previously mentioned studies suggest that conifer chitinases play their most significant roles in defense against pathogens. The antifungal activity of a class IV chitinase from *P. abies* was tested recently by Ubhayasekera *et al.* (2009) who confirmed that this conifer chitinase strongly inhibits *H. annosum* growth. In the same *P. abies*-*H. annosum* pathosystem, expression of chitinase genes was found to be higher in resistant *P. abies* clones than in susceptible clones a few days after infection with *H. annosum* (Hietala *et al.* 2004; Fossdal *et al.* 2006, 2007). Hietala *et al.* (2004) monitored *H. annosum* colonization and chitinase transcript levels in *P. abies* clones differing in disease resistance and concluded that chitinase gene expression is correlated with the resistance mechanism of *P. abies*. The high constitutive levels of chitinases in *P. abies* may signify a role in releasing fungal cell wall elicitors at the onset of infection (Fossdal *et al.* 2006, 2007).

Sequencing of a cDNA library constructed from *P. sulphurascens*-infected DF seedling roots showed that the chitinase gene family is one of the largest constituents of the DF cDNA library (Islam *et al.* 2010). At least three class II and six class IV chitinase genes were identified from DF seedlings. Real-time PCR analyses showed significant differential expression patterns of these genes locally in root tissues and systemically in needle tissues after fungal inva-

Table 1 Functions and biological processes of conifer chitinases.

Origin	Chitinase type	Functions/biological processes	References
Pseudotsuga menziesii	Class IV and II	Defense against pathogen	Islam *et al.* 2010a
Pseudotsuga menziesii	Endochitinase		Robinson *et al.* 2000; Zamani *et al.* 2003; Sturrock *et al.* 2007; Islam *et al.* 2009
Pinus monticola	Class IV		Liu *et al.* 2005
Pinus taeda	Class II		Davis *et al.* 2002
Picea abies	Class II		Jøhnk *et al.* 2005
Picea abies	Class I, II, IV		Hietala *et al.* 2004
Picea glauca	Chitinase		Nagy *et al.* 2004
Picea abies	Chitinase		Asiegbu *et al*. 1993; Sharma *et al.* 1993; Fossdal *et al*. 2006, 2007
Pinus sylvestris	Chitinase		Hodge *et al.* 1995
Pinus contorta	Chitinase		Nsolomo and Woodward 2007
Pinus sylvestris	Chitinase		Asiegbu *et al*. 1995, 2005; Nsolomo and Woodward 2007
Pinus nigra	Chitinase		Nsolomo and Woodward 2007
Picea abies	Chitinase		Kozlowski and Métraux 1998; Kozlowski *et al.* 1999
Pinus sylvestris	Chitinase IV		Adomas *et al.* 2007, 2008
Pinus taeda	Chitinase		Popp *et al.* 1997
Pinus strobes	Class II	Defense against wounding	Wu *et al.* 1999
Picea abies	Class II, IV		Hietala *et al.* 2004; Fossdal *et al.* 2006, 2007
Picea glauca	Class IV		Dong and Dunstan 1997
Picea sitchensis	Class IV, chitinase		Ralph *et al.* 2006; Lippert *et al.* 2007
Pinus taeda	Class IV, chitinase	Defense against drought	Chang *et al.* 1996; Lorenz *et al.* 2006
Pinus halepensis	Class I		Sathyan 2004
Pinus taeda	Class I		Sathyan 2004
Picea abies	Chitinase		Nagy *et al.* 2004
Picea glauca	Class IV		Dong and Dunstan 1997
Picea abies	Class II, IV	Defense against overwintering	Jarząbek *et al.* 2009
Picea pungens	Class II, IV		Jarząbek *et al.* 2009
Pseudotsuga menziesii	Endochitinase		Zamani *et al.* 2003
Picea sitchensis	Class IV	Defense against insects	Lippert *et al.* 2007
Picea sitchensis	Class IV, chitinase		Ralph *et al.* 2006
Pinus densiflora	Endochitinase	Defense against nematodes	Shin *et al*. 2009
Picea abies	Class I	Mycorrhizal symbiosis	Salzer *et al.* 1997a, 1997b
Pinus sylvestris	Chitinase		Hodge *et al.* 1995
Cryptomeria japonica	Class IV	Pollen allergen	Fujimura *et al.* 2005, 2007
Picea abies	Class IV	Growth and development	Wiweger *et al.* 2003
Picea glauca	Class IV		Dong and Dunstan 1997
Araucaria angustifolia	Class IV		dos Santos *et al.* 2006, 2008
Pinus sylvestris	Chitinase	Program cell death	Pirttilä *et al.* 2002
Picea abies	Class IV		Wiweger *et al.* 2003
Pinus storbus	Class II	Chitosan-induced	Wu *et al.* 1997
Pinus elliottii	Class II		Mason and Davis 1997
Pinus taeda	Chitinase		Popp *et al.* 1997; Davis *et al.* 2002
Pinus taeda	Class I, II, IV	Salicylic acid-induced	Davis *et al.* 2002
Picea abies	Chitinase		Kozlowski and Métraux 1998
Pinus taeda	Class I, IV	Jasmonic acid-induced	Davis *et al.* 2002
Pinus monticola	Class IV	Methyl jasmonate-induced	Liu *et al.* 2005
Picea abies	Chitinase		Kozlowski *et al.* 1999
Picea abies	Class I	Chitotetraose/chitin-induced	Salzer *et al.* 1997a, 1997b
Pinus monticola	Class IV	Inhibitor-induced	Liu *et al.* 2005
Cycus revoluta	Class V	Transglycosylation activity	Taira *et al.* 2009

sion. Western immunoblot data also showed significant accumulation of a class IV chitinase in *P. sulphurascens*-infected DF seedlings (Islam *et al.* 2010). Previously, an endochitinase-like protein was reported as occurring in the roots of 11- and 25-year-old DF trees that were naturally infected with *Armillaria ostoyae*. This protein was also found to be upregulated in DF plants infected with *P. sulphurascens* (formerly *P. weirii*) (Robinson *et al.* 2000; Zamani *et al.* 2003). Western immunoblots also revealed that the apoplastic fluid of DF roots and needles contained multiple ECP isoforms with isoelectric points ranging from 5.3 to 5.8 and molecular masses of 27–30 kDa (Zamani *et al.* 2003). Sturrock *et al.* (2007) demonstrated that this ECP protein was significantly upregulated in *P. sulphurascens*-infected DF seedling roots (**Fig. 6**).

In *P. elliottii* seedlings, multiple chitinase homologs accumulated after challenge by the fungal pathogen *Fusarium subglutinans* Woll. & Rein. These chitinase isoforms were also induced by potential signaling molecules identified from angiosperms (Davis *et al.* 2002). In *P. abies* seedling roots, chitinase accumulation was increased three days after inoculation with *Pythium* sp. (Sharma *et al.* 1993).

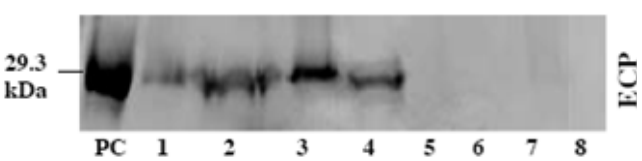

Fig. 6 Western immunoblot showing the accumulation of an endochitinase protein in DF seedlings. The root (lanes 1 to 4) and needle (lanes 5 to 8) samples of young Douglas-fir seedlings collected from uninfected controls at 12 days post inoculation (dpi) and from *Phellinus sulphurascens*-infected plants at 2, 7, and 12 dpi. PC-positive controls obtained from Douglas-fir needles for ECP. Lanes 1 and 5, uninfected controls; lanes 2 and 6, 2 dpi; lanes 3 and 7, 7 dpi; and lanes 4 and 8, 12 dpi (modified from Sturrock *et al.* 2007).

Chitinase activity was also increased in *P. abies* seedlings infected with pathogenic oomycetes (Kozlowski and Métraux 1998) and both local and systemic increases in chitinase activity were recorded after inoculation with the root die-back fungus *Rhizoctonia* (Nagy *et al.* 2004). Asiegbu *et al.* (1999) reported that following adhesion and cellular penetration by the pathogen, several pathogenesis-related proteins, including chitinase and glucanase, are induced in roots of *P. abies* inoculated with *Fusarium avenaceum* (Corda ex Fries) Sacc. Liu *et al.* (2005) reported that two isoforms of a class IV chitinase were differentially regulated in slow-canker-growth resistant and susceptible seed families of *P. monticola*. A 27-kDa chitinase isozyme (PmCh4A) accumulated in both susceptible and slow-canker-growth resistant seedlings after infection by *Cronartium ribicola* J. C. Fisch., while a 26-kDa chitinase isozyme was expressed specifically in slow-canker-growth resistant seedlings. Wounding treatment also induced expression of this protein suggesting that *P. monticola* chitinases play important defense roles and can be used in marker-assisted selection in forest breeding (Liu *et al.* 2005).

Recent work by Stefani *et al.* (2010), which considered the environmental impact of transformed trees, showed that an endochitinase-transformed white spruce had no negative effect on soil fungal biomass and ectendomycorrhizal symbiosis. While these results are encouraging, further research on transformed trees, including the use of large-scale field trials, is needed.

Conifer chitinases in defense against insects

Insect feeding can have major ecological and economic impacts on both natural and planted conifer forests. Resistance to insect attack in conifers is a major focus of current forest health research programs. Chitinases may perform numerous roles in the defense against insect pests or against insect-associated fungi. There is evidence that a class IV chitinase was differentially induced in white pine weevil (*Pissodes strobe* W. D. Peck)-infested *Picea sitchensis* (Bong.) Carr. bark (Lippert *et al.* 2007). A poplar chitinase is also induced during infestation by gypsy moth (*Lymantria dispar* L.) larvae (Lawrence and Novak 2006). These data suggest that chitinases play a role in defense against insects. Plant-derived chitinase enzyme can be active in the insect gut and can have detrimental effects in the development of insect herbivores (Chen *et al.* 2008; Howe and Jander 2008).

Conifer chitinases in wound stress

Conifer chitinases can be induced subsequent to wound or mechanical damage. For example, in *P. abies* wounding alone resulted in a clear gradient in the expression of chitinases (PaChi4 and PaChi2) with the highest levels immediately adjacent to the inoculation point (Hietala *et al.* 2004). In contrast to inoculation, the maximum induction levels of the two genes were observed three days after wounding. Chitinase PaChi2 has high similarity to an extracellular class II chitinase, Pschi4, of *Pinus strobus* that is induced by chitosan and wounding (Wu *et al.* 1997, 1999). Dong and Dunstan (1997) also reported that wounding enhanced chitinase- and glucanase-related gene expression in *P. glauca*.

Conifer chitinases in drought stress

Drought has been demonstrated to induce multiple chitinases in *P. taeda* (Lorenz *et al.* 2006). Sathyan (2004) also reported that water stress induced chitinases in *P. taeda* and *P. halepensis*, while Nagy *et al.* (2004) reported that several isoforms of peroxidase and chitinase were differentially accumulated in *Picea abies* after exposure to drought and the fungal pathogen *Rhizoctonia*. In a contrary finding, a chitinase isoform was observed to be down-regulated in *P. taeda* in response to water-deficit stress (Chang *et al.* 1996); however, this protein-coding gene is not the same one reported by the previous authors. Fossdal *et al.* (2006, 2007) found that a combination of pathogen infection and drought stress lead to a specific elevation of chitinases in *P. abies*. Similar to gymnosperms, angiosperm chitinases have also been found to be expressed under drought stress (Bray 2004).

Conifer chitinases in frost/overwintering stresses

Antifreeze proteins (AFPs) refer to a class of polypeptides that enhance the ability of certain organisms including vertebrates, plants, fungi and bacteria to endure freezing environments. These proteins permit the survival of cells in subzero environments. AFPs bind to small ice crystals to inhibit expansion, protrusion, and recrystallization of ice that would otherwise be fatal (Kuiper 2001; Jorov *et al.* 2004). In most plants, freezing tolerance varies among plant organs, and is strongly correlated with seasons of the year, thereby changing in the course of plant development (Larcher 2003). Conifer chitinases possess potent antifreeze and cryoprotective properties. For example, a 27-kDa apoplastic chitinase-like protein (AP27) was identified from *P. abies* and *Picea pungens* Engelm. Needles that was shown to modify the growth of ice and thermal hysteresis. The key feature of the N-terminal sequence of this protein is the presence of many hydrophilic residues, including serine, threonine, aspartic acid and glutamine (Jarząbek *et al.* 2009). These N-terminal amino acid residues can directly bind ice crystals and, as a result, limit ice growth in living tissues (Davies *et al.* 2002; Leinala *et al.* 2002). A similar type of apoplastic endochitinase protein collected from *P. sulphurascens*-infected winter *P. menziesii* needles showed evidence of its higher accumulation in winter months (Zamani *et al.* 2003). The N-terminal sequences of this protein revealed high sequence homology to class II and/or class IV chitinases from other conifer species including *P. abies*, *P. glauca*, and *P. monticola.* As with conifer chitinases, a number of other PR proteins secreted into the apoplast during cold acclimation are thought to be responsible for disease resistance (Ekramoddoullah *et al.* 1995, 2001; Matheus *et al.* 2003). Interestingly, some cold-induced PR proteins, including chitinases, display both antifungal and antifreeze activities, suggesting a dual function of these proteins in protecting plants from overwintering and other biotic and abiotic stresses (Hon *et al.* 1995; Kuwabara and Imai 2009). However, the signaling pathway for cold-induced disease resistance is currently unknown but can be independent of pathogen-induced defense mechanisms (Kuwabara and Imai 2009).

Conifer chitinases in other stresses

The available data suggest that plant chitinases play a role in combating a variety of stresses. For example, a basic chitinase (ChitiWb1) encoding gene significantly increased in leaves and cultured cells of wing bean treated with NaCl, KCl, $CaCl_2$, mannitol or saccharose (Tateishi *et al.* 2001). In tobacco, extracellular chitinases were also increased significantly under salt stress (Dani *et al.* 2005). Dong and Dunstan (1997) reported that wounding and other stresses such as drying and flooding enhanced chitinase- and glucanase-related gene expression in *P. glauca*. These data infer that conifer chitinases may play important defensive roles against most abiotic stresses, aside from their major roles in pathogen defense and plant development.

Conifer chitinases in programmed cell death

Several studies have demonstrated that in *P. glauca* and *P. abies*, class IV chitinases are expressed during embryo development and have been associated with programmed cell death (PCD; Dong and Dunstan 1997; Dyachok *et al.* 2000, 2002; Wiweger *et al.* 2003), possibly through hydrolyzing or releasing oligosaccharides related to cell-to-cell signaling. Class IV chitinase genes have also been impli-

cated in PCD in other plants such as carrot and *Arabidopsis*, possibly by acting on arabinogalactan proteins (van Hengel *et al.* 1998; Passarinho *et al.* 2001), and might also be involved in transition from polyembryonal masses to somatic embryos by direct or indirect activation of PCD (Wiweger *et al.* 2003). The question is whether the involvement of conifer chitinases in PCD is only related to growth and development of embryos or whether they activate some hypersensitive responses associated with plant defense at the early stage of plant development. Further investigation is required to better understand the association of conifer chitinases in PCD.

Conifer chitinases in pollen allergens

A class IV chitinase (CJP-4) has been isolated from *C. japonica* pollen. The purified protein displayed the ability to bind IgE from all patients tested. The CJP-4 is a 34-kDa protein, displaying endochitinase activity that cross-reacts with latex allergens (Fujimura *et al.* 2005, 2007). Similarly, there are some other conifer PR proteins that also display allergic behavior, including thaumatin-like proteins and PR10 proteins (Futamura *et al.* 2002; Liu *et al.* 2003; Fujimura *et al.* 2005, 2007). According to Fujimura *et al.* (2005), the CJP-4 and other chitinases may act as causative allergens of the cross-reactivity in *C. japonica* pollinosis and oral allergy syndrome. There is evidence that angiosperm pollen chitinases are also involved in allergic reactions. For example, a 32-kDa IgE-binding green bean protein was strongly induced by ethylene treatment. The protein, designated as PvChI, was identified as a class I chitinase closely related to the major avocado allergen Prs a 1 (Sánchez-Monge *et al.* 2000).

Conifer chitinases in growth and development

Chitinase-like proteins have long been proposed to play roles in normal plant growth and development (De Jong *et al.* 1992; Collinge *et al.* 1993; Dong and Dunstan *et al.* 1997; Zhong *et al.* 2002). The expression of plant chitinase genes in the absence of pathogen attack or other stress conditions has been studied in many plant tissues, including those of conifers (Collinge *et al.* 1993; Høj and Fincher 1995; Dong and Dunstan 1997; Dyachok *et al.* 2002; Wiweger *et al.* 2003; dos Santos *et al.* 2008). This expression suggests that chitinases are involved in plant growth and development. The non-defensive role of plant chitinases was initially proposed from a study of somatic embryo development in carrot plant (De Jong *et al.* 1992; Kragh *et al.* 1996). In recent years, embryogenic cultures of *P. abies* have been used extensively for studying the regulation of embryo development (von Arnold *et al.* 2002). The developmental pathway of somatic embryogenesis of *P. abies* involves proliferation of proembryogenic masses, somatic embryo transition and further development of somatic embryos (Wiweger *et al*. 2003). The importance of chitinases in *P. abies* embryogenesis *in vitro* was demonstrated by stimulating early somatic embryo development in the presence of exogenously supplied chitinase which was derived from *Streptomyces griseus* Waks. & Henr. (Egertsdotter and von Arnold 1995, 1998; Dyachok *et al.* 2002) or from a preconditioned medium of embryogenic cultures (Egertsdotter *et al.* 1993). Furthermore, the expression of chitinase-like proteins appears to be developmentally regulated during growth and development in other conifers. For example, in *P. glauca* the transcript of a chitinase (*PgChi*- 1) is highly abundant in embryogenic tissues. The expression level was further increased between the filamentous stage of development and in fully developed cotyledonary embryos (Dong and Dunstan 1997). It has been suggested that during *in vitro* somatic embryogenesis extracellular chitinases are able to cleave the glycosidic bonds of glucosamine and N-acetylglucosamine residues in the sugar moiety of arabinogalactan proteins (AGPs; van Hengel *et al.* 2001) generating oligosaccharide signal molecules essential for early stages of plant embryogenesis (Malinowski and Filipecki 2002). Another study also reported that the embryogenic tissues and nonembryogenic calli of *Pinus caribaea* Morel. produce proteins of 48 or 56 and 25 kDa, respectively. All of these proteins cross-react with several classes of tobacco chitinases. These chitinase-like proteins showed potential chitinase activity on SDS-PAGE gels overlaid with glycol chitin as a synthetic substrate. However, when an AGP fraction from embryogenic tissues substitutes for glycol chitin on gels, only the 48-kDa embryo-specific chitinase-like protein acts on this substrate suggesting that an interaction between this protein and a specific set of AGPs might exist within embryogenic tissues of *P. caribaea* (Domon *et al.* 2000). There is also evidence that extracellular chitinase protein expressed in the endosperm of carrot rescues somatic embryos of the carrot ts11 variant (van Hengel *et al.* 1998).

It is assumed that chitinases secreted in *P. abies* and carrot cultures can degrade lipo-chitooligosaccharides (LCOs). In both *P. abies* and carrot embryogenic systems, 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), which can promote the development of pro-embryogenic masses (PEMs) from small cell aggregates in *P. abies* (Egertsdotter and von Arnold 1998; Dyachok *et al.* 2000). It is not yet confirmed that secreted chitinases can degrade LCOs in a similar way as plant chitinases hydrolyze the rhizobial Nod factors (Staehelin *et al.* 1994, 1995). However, data suggest that chitinases are involved in the production of plant signal molecules that are similar to the rhizobial Nod factors. For example, the promotion of somatic embryo development in carrot by endochitinase EP3 could be mimicked by rhizobial LCOs (De Jong *et al.* 1992). The EP3 chitinase colocalizes with AGPs in developing seeds, and it was shown to cleave AGPs *in vitro* (van Hengel *et al.* 2001). Furthermore, dos Santos *et al*. (2008) observed the somatic embryos of a superior genotype showed higher chitinase expression and concluded that chitinases can be used as an indicator of a genotypic superiority for the development of somatic embryogenesis in *Araucaria angustifolia* (Bertol.) Kuntz.

Recently mutations in chitinase-like genes have been obtained in *Arabidopsis* to probe the developmental role of plant chitinases. A chitinase-like gene (*AtCTL1*) encoding protein AtCTL1 caused ectopic deposition of lignin and aberrant shapes of cells with incomplete cell walls in the pith of inflorescence stems. Consistent with its ubiquitous expression pattern, mutation of the *AtCTL1* gene affected many aspects of plant growth and development, including exaggerated hook curvature, reduced length and increased diameter of hypocotyls in dark-grown seedlings, and reduced root length and increased number of root hairs in light-grown seedlings. These results suggest that *AtCTL1* is essential for normal plant growth and development in *Arabidopsis* (Zhong *et al.* 2002). Taira *et al.* (2009) reported a class V chitinase from a *Cycas revoluta* which showed transglycosylation activity.

Conifer chitinases in biocontrol

Researchers interested in new biopesticides have given considerable attention to chitinases (Brown 1998; Goodday 1999; Herrera-Estrella and Chet 1999; Karasuda *et al.* 2003; Chung and Kim 2007). There is evidence that a class IV chitinase from a yam (*Dioscorea opposita* Thunb.) can effectively control the powdery mildew of strawberries (Karasuda *et al.* 2003). It is also suggested that *Escherichia coli* is able to produce recombinant chitinase in the soil that can control the pathogenesis by *F. oxysporum* without colonization (Chung and Kim 2007). Since chitinases are one of the dominant protein families in conifer systems, there is a great potential to explore chitinase-based products from conifers that could be commercially produced using recombinant protein technology. For example, *E. coli* expressing

an endochitinase gene can effectively control *Fusarium* wilt of cucumber. Since insects are too expensive to be a commercial source of chitinase (Brown 1998), conifers could be an option as a source of chitinases. Biological control incorporating the use of a biodegradable enzyme like chitinase would be more environmentally sound than conventional pesticide applications.

Conifer chitinases in biofuel production

Many living organisms use complex networks of fibrous and crystalline polysaccharides to maintain their structural integrity. Enzymatic conversion of the most recalcitrant of these polysaccharides is of great biological and economic importance (Eijsink *et al.* 2008). In plants, the major structural polysaccharide is cellulose [β(1→4)linked glucose], whereas non-plants such as insects, crustaceans and fungi employ chitin [β(1→4)linked *N*-acetyl glucosamine], which occurs in two major forms, α-chitin and β-chitin. Cellulose and chitin are the most abundant biopolymers in the terrestrial and marine environments, respectively. In nature, degradation of cellulosic or chitinous biomass is achieved by mixtures of hydrolytic exo- and endo-acting enzymes that act in a synergistic manner (Horn *et al.* 2006; Merino and Cherry 2007). Eijsink *et al.* (2008) claimed that chitinase could be used for the future development of biomass conversion. In conifers, the abundance of different chitinases indicates potential for future biomass conversion research.

CONCLUSION

Our understanding of the role of conifer chitinases in defense against pests and pathogens lags behind that pertaining to chitinases involved in defense against pathogens affecting short-lived angiosperm crop plants. To date, a variety of studies have confirmed that conifer chitinases play a significant role in many aspects of plant protection against biotic and abiotic stresses. They also function in plant growth and development. Despite constitutive expression of chitinases in conifer plants, chitinases have also been found to be induced locally and systemically by different stressors. Signal molecules such as salicylic acid, jasmonic acid and ethylene play key roles in chitinase induction in conifer systems. Several studies have significantly contributed to our understanding of this ubiquitous protein family in conifer plants, providing more knowledge of chitinase structural properties, antifungal activities, substrate specificity and catalytic mechanisms. The available literature also reveals that conifer chitinases serve non-defensive functions during somatic embryogenesis, somatic embryo rescues and PCD. Several recent lines of evidence have substantiated the biotechnological potential of conifer chitinases to counter fungal diseases. Further chitinase genomics and proteomics research will enhance our understanding of structural diversity, substrate specificities, regulations and isoform specific functions of conifer chitinases. There is also a need to conduct research to realize the potential applications of conifer chitinases in biocontrol and biofuel production for future generations.

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REFERENCES

Adomas A, Heller G, Li G, Olson Å, Chu T-M, Osborne J, Craig D, van Zyl L, Wolfinger R, Sederoff R, Dean RA, Stenlid J, Finlay R, Asiegbu FO (2007) Transcript profiling of a conifer pathosystem: Response of *Pinus sylvestris* root tissues to pathogen (*Heterobasidion annosum*) invasion. *Tree Physiology* **27**, 1441-1458

Adomas A, Heller G, Olson Å, Osborne J, Karlsson M, Nahalkova J, van Zyl L, Sederoff R, Stenlid J, Finlay R, Asiegbu FO (2008) Comparative analysis of transcript abundance in *Pinus sylvestris* after challenge with a saprotrophic, pathogenic or mutualistic fungus. *Tree Physiology* **28**, 885-897

Andersen OA, Dixon MJ, Eggleston IM, van Aalten DM (2005) Natural product family 18 chitinase inhibitors. *Natural Product Reports* **22**, 563-579

Araki T, Torikata T (1995) Structural classification of plant chitinases: Two subclasses in class I and class II chitinases. *Bioscience, Biotechnology and Biochemistry* **59**, 336-338

Asiegbu FO, Daniel G, Johansson M (1993) Studies on the infection of Norway spruce roots by *Heterobasidion annosum*. *Canadian Journal of Botany* **71**, 1552-1561

Asiegbu FO, Denekamp M, Daniel G, Johansson M (1995) Immuno-cytochemical localization of pathogenesis-related proteins in roots of Norway spruce infected with *Heterobasidion annosum*. *European Journal of Forest Pathology* **25**, 169-178

Asiegbu FO, Kacprzak M, Daniel G, Johansson M, Stenlid J, Manka M (1999) Biochemical interactions of conifer seedling roots with *Fusarium* spp. *Canadian Journal of Microbiology* **45**, 923-935

Asiegbu FO, Nahalkova J, Li G (2005) Pathogen-inducible cDNAs from the interaction of the root rot fungus *Heterobasidion annosum* with Scots pine (*Pinus sylvestris* L.). *Plant Science* **168**, 365-372

Beintema JJ (1994) Structural features of plant chitinases and chitin-binding proteins. *FEBS Letters* **350**, 159-163

Benhamou N, Joosten MHAJ, De Wit PJGM (1990) Subcellular localization of chitinase and of its potential substrate in tomato root tissues infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Plant Physiology* **92**, 1108-1120

Bhattacharya D, Nagpure A, Gupta RK (2007) Bacterial chitinases: Properties and potential. *Critical Reviews in Biotechnology* **27**, 21-28

Bishop JG, Dean AM, Mitchell-Olds T (2002) Rapid evolution in plant chitinases: Molecular targets of selection in plant-pathogen co-evolution. *Proceedings of the National Academy of Science USA* **97**, 5322-5327

Boller T, Metraux JP (1988) Extracellular localization of chitinase in cucumber. *Physiological and Molecular Plant Pathology* **33**, 11-16

Bonello P, Gordon TR, Herms DA, Wood DL, Erbilgin N (2006) Nature and ecological implications of pathogen-induced systemic resistance in conifers: A novel hypothesis. *Physiological and Molecular Plant Pathology* **68**, 95-104

Bray EA (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. *Journal of Experimental Botany* **55**, 2331-241

Brown J (1998) Possible new biopesticide. *Agrichemical and Environmental News* **146**, 4-5

Brunner F, Stintzi A, Fritig B, Legrand M (1998) Substrate specificities of tobacco chitinases. *The Plant Journal* **14**, 225-234

Burketová L, Štillerová K, Feltlová M (2003) Immunohistological localization of chitinase and β-1,3-glucanase in rhizomania-diseased and benzothiadiazole treated sugar beet roots. *Physiological and Molecular Plant Pathology* **63**, 47-54

Chang S, Puryear J, Dias MADL, Funkhouser EA, Newton RJ, Cairney J (1996) Cloning of DNA for chitinase homologue which lacks chitin binding sites and is down regulated by water stress and wounding. *Plant Molecular Biology* **31**, 693-699

Chen H, Gonzales-Vigil E, Howe GA (2008) Action of plant defensive enzymes in the insect midgut. In: Schaller A (Ed) *Induced Plant Resistance to Herbivory*, Springer Publishing Co., The Netherlands, pp 271-284

Chung S, Kim S-D (2007) *Escherichia coli* can produce recombinant chitinase in the soil to control the pathogenesis by *Fusarium oxysporum* without colonization. *Journal of Microbiology and Biotechnology* **17**, 474-480

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. *The Plant Journal* **3**, 31-40

Dani V, Simon WJ, Duranti M, Croy RRD (2005) Changes in the tobacco leaf apoplast proteome in response to salt stress. *Proteomics* **5**, 737-745

Davies PL, Baardsnes J, Kuiper M, Walker VK (2002) Structure and function of antifreeze proteins. *Philosophical Translations of Royal Society London B. Biological Sciences* **357**, 927-935

Davis JM, Wu H, Cooke JE, Reed JM, Luce KS, Michler CH (2002) Pathogen challenge, salicylic acid, and jasmonic acid regulate expression of chitinase gene homologs in pine. *Molecular Plant-Microbe Interactions* **15**, 380-387

De Jong AJ, Cordewener J, Lo Schiavo F, Terzi M, Vandekerckhove J, Van Kammen A, De Vries SC (1992) A carrot somatic embryo mutant is rescued by chitinase. *The Plant Cell* **4**, 425-433

Domon J-M, Neutelings G, Roger D, Daid A, David H (2000) A basic chitinase-like protein secreted by embryogenic tissues of *Pinus caribaea* acts on arabinogalactan proteins extracted from the same cell lines. *Journal of Plant Physiology* **156**, 33-39

Dong J, Dunstan DI (1997) Endochitinase and β-1,3-glucanase genes are developmentally regulated during somatic embryogenesis in *Picea glauca*. *Planta* **201**, 189-194

dos Santos ALW, Steiner N, Guerra MP, Zoglauer K, Moerschbacher BM (2008) Somatic embryogenesis in *Araucaria angustifolia*. *Biologia Plantarum* **52**, 195-199

dos Santos ALW, Wiethölterb N, Gueddaria NEE, Moerschbacher BM (2006) Protein expression during seed development in *Araucaria angustifolia*: Transient accumulation of class IV chitinases and arabinogalactan proteins. *Physiologia Plantarum* **127**, 138-148

Dyachok JV, Tobin AE, Price NPJ, von Arnold S (2000) Rhizobial Nod factors stimulate somatic embryo development in *Picea abies*. *Plant Cell Reports* **19**, 290-297

Dyachok JV, Wiweger M, Kenne L, von Arnold S (2002) Endogenous Nod-factor-like signal molecules promote early somatic embryo development in Norway spruce. *Plant Physiology* **128**, 523-533

Egertsdotter U, Mo LH, von Arnold S (1993) Extracellular proteins in embryogenic suspension cultures of Norway spruce (*Picea abies*). *Physiologia Plantarum* **88**, 315-321

Egertsdotter U, von Arnold S (1995) Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). *Physiologia Plantarum* **93**, 334-345

Egertsdotter U, von Arnold S (1998) Development of somatic embryos in Norway spruce. *Journal of Experimental Botany* **49**, 155-162

Eijsink VGH, Vaaje-Kolstad G, Vårum KM, Horn SJ (2008) Towards new enzymes for biofuels: Lessons from chitinase research. *Trends in Biotechnology* **26**, 228-235

Ekramoddoullah AKM, Taylor D, Hawkins BJ (1995) Characterization of a fall protein of sugar pine and detection of its homologue associated with frost hardiness of western white pine needles. *Canadian Journal of Forest Research* **25**, 1137-1147

Ekramoddoullah AKM, Yu X, Sturrock R, Zamani A, Taylor D (2001) Detection and seasonal expression pattern of a pathogenesis- related protein (PR-10) in Douglas-fir (*Pseudotsuga menziesii*) tissues. *Physiologia Plantarum* **110**, 240-247

Flach J, Pilet P-E, Jolles P (1992) What's new in chitinase research. *Experientia* **48**, 701-716

Fossdal CG, Hietala AM, Kvaalen H, Solheim H (2006) Changes in host chitinase isoforms in relation to wounding and colonization by *Heterobasidion annosum*: Early and strong defense response in 33-year-old resistant Norway spruce clone. *Tree Physiology* **26**, 169-77

Fossdal CG, Nagy NE, Johnsen Ø, Dalen LS (2007) Local and systematic stress responses in Norway spruce: Similarities in gene expression between a compatible pathogen interaction and drought stress. *Physiological and Molecular Plant Pathology* **70**, 161-173

Franceschi VR, Krokene P, Christiansen E, Krekling T (2005) Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist* **167**, 353-376

Fujimura T, Shigeta S, Suwa T, Kawamoto S, Aki T, Masubuchi M, Hayashi T, Hide M, Ono K (2005) Molecular cloning of a class IV chitinase allergen from Japanese cedar (*Cryptomeria japonica*) pollen and competitive inhibition of its immunoglobulin E-binding capacity by latex C-serum. *Clinical and Experimental Allergy* **35**, 234-243

Fujimura T, Futamura N, Midoro-Horiuti T, Togawa A, Goldblum RM, Yasueda H, Saito A, Shinohara K, Masuda K, Kurata K, Sakaguchi M (2007) Isolation and characterization of native Cry j 3 from Japanese cedar (*Cryptomeria japonica*) pollen. *Allergy* **62**, 547-553

Futamura N, Mukai Y, Sakaguchi M, Yasueda H, Inouye S, Midoro-Horiuti T, Goldblum RM, Shinohara K (2002) Isolation and characterization of cDNAs that encode homologs of a pathogenesis-related protein allergen from *Cryptomeria japonica*. *Bioscience, Biotechnology and Biochemistry* **66**, 2495-2500

Gomez L, Allona I, Casado R, Aragoncillo C (2002) Seed chitinases. *Seed Science Research* **12**, 217-230

Gooday GW (1999) Aggressive and defensive roles for chitinases. *Experimentia Supplementum* **87**, 157-169

Graham LS, Sticklen MB (1994) Plant chitinases. *Canadian Journal Botany* **72**, 1057-1083

Hamel F, Boivin R, Tremblay C, Bellemare G (1997) Structural and evolutionary relationship among chitinases of flowering plants. *Journal of Molecular Evolution* **44**, 614-624

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemistry Journal* **293**, 781-788

Herrera-Estrella A, Chet I (1999) Chitinases in biological control. *Experimentia Supplementum* **87**, 171-84

Hietala AM, Kvaalen H, Schmidt A, Jøhnk N, Solheim H, Fossdal CG (2004) Temporal and spatial profiles of chitinase expression by Norway spruce in response to bark colonization by *Heterobasidion annosum*. *Applied and Environmental Microbiology* **70**, 3948-3953

Himmel M, Ding S-Y, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* **315**, 804-807

Hodge A, Alexander IJ, Gooday GW (1995) Chitinolitic activities of *Eucalyptus pilularis* and *Pinus sylvestris* root systems challenged with mycorrhizal and pathogenic fungi. *New Phytologist* **131**, 255-261

Høj PB, Fincher GB (1995) Molecular evolution of plant β-glucan endohydrolases. *Plant Journal* **7**, 367-379

Hollis T, Monzingo AF, Bortone K, Ernst S, Cox R, Robertus JD (2000) The X-ray structure of a chitinase from the pathogenic fungus *Coccidioides immitis. Protein Science* **9**, 544-551

Holopainen JK, Heijar J, Nerg A-M, Vuorine M, Kainulainen P (2009) Potential for the use of exogenous chemicals elicitors in disease and insect pest management of conifer seedling production. *The Open Forest Science Journal* **2**, 17-24

Hon WC, Griffith M, Mlynarz A, Kwok YC, Yang DS (1995) Antifreeze proteins in winter rye are similar to pathogenesis related proteins. *Plant Physiology* **109**, 879-889

Horn SJ, Sørbotten A, Synstad B, Sikorski P, Sørlie M, Varum KM, Eijsink VGH (2006) Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*. *FEBS Journal* **273**, 491-503

Howe GA, Jander G (2008) Plant immunity to insect herbivores. *Annual Review of Plant Biology* **59**, 41-66

Iseli B, Armand S, Boller T, Neuhaus JM, Henrissat B (1996) Plant chitinases use two different hydrolytic mechanisms. *FEBS Letters* **382**, 186-188

Islam MA, Sturrock RN, Holmes TA, Ekramoddoullah AKM (2009) Ultrastructural studies of *Phellinus sulphurascens* infection of Douglas-fir roots and immunolocalization of host pathogenesis-related proteins. *Mycological Research* **113**, 700-712

Islam MA, Sturrock RN, Williams HL, Ekramoddoullah AKM (2010) Identification, characterization and expression analyses of class II and IV chitinase genes from Douglas-fir seedlings infected by *Phellinus sulphurascens*. *Phytopathology* **100**, 356-366

Jarząbek M, Pukacki PM, Nuc K (2009) Cold-regulated proteins with potent antifreeze and cryoprotective activities in spruces (*Picea* spp.). *Cryobiology* **58**, 268-274

Jøhnk N, Hietala AM, Fossdal CG, Collinge DB, Newman MA (2005) Defense-related genes expressed in Norway spruce roots after infection with the root rot pathogen *Ceratobasidium bicorne* (anamorph: *Rhizoctonia* sp.). *Tree Physiology* **25**, 1533-43

Jorov A, Zhorov BS, Yang DSC (2004) Theoretical study of interaction of winter flounder antifreeze protein with ice. *Protein Science* **13**, 1524-1537

Kado T, Yoshimaru H, Tsumura Y, Tachida H (2003) DNA variation in a conifer, *Cryptomeria japonica* (Cupressaceae *sensu lato*). *Genetics* **164**, 1547-1559

Karasuda S, Tanaka S, Kajihara H, Yamamoto Y, Koga D (2003) Plant chitinase as a possible biocontrol agent for use instead of chemical fungicides. *Bioscience, Biotechnology, and Biochemistry* **67**, 221-224

Karlsson M (2005) Transcriptional responses during the pathogenic interaction between *Heterobasidion annosum* s. l. and conifers. PhD thesis, Department of Forest Mycology and Pathology, Swedish University of Agriculture, Uppsala, Sweden, 34 pp

Karlsson M, Olson A, Stenlid J (2003) Expressed sequences from the basidiomycetous tree pathogen *Heterobasidion annosum* during early infection of Scots pine. *Fungal Genetics and Biology* **39**, 51-59

Kasprzewska A (2003) Plant chitinases–regulation and function. *Cellular and Molecular Biology Letters* **8**, 809-824

Koga D (2005) Application of chitinases in agriculture. *Journal of Metals, Materials and Minerals* **15**, 33-35

Kozlowski G, Buchala A, Métraux J-P (1999) Methyl jasmonate protects Norway spruce [*Picea abies* (L.) Karst.] seedlings against *Pythium ultimum* Trow. *Physiological and Molecular Plant Pathology* **55**, 53-58

Kozlowski G, Métraux J-P (1998) Infection of Norway spruce (*Picea abies* (L.) Karst.) seedlings with *Pythium irregulare* Buism. and *Pythium ultimum* Trow: Histological and biochemical responses. *European Journal Plant Pathology* **104**, 225-234

Kragh KM, Hendriks T, De Jong AJ, Schiavo FL, Bucherna N, Højrup P, Mikkelsen JD, De Vries SC (1996) Characterization of chitinases able to rescue somatic embryos of the temperature-sensitive carrot variants. *Plant Molecular Biology* **31**, 631-645

Kuiper M (2001) A theoretical model of a plant antifreeze protein from *Lolium perenne. Biophysical Journal* **81**, 3560-3565

Kusumi J, Tsumura Y, Yoshimaru H, Tachida H (2002) Molecular evolution of nuclear genes in Cupressacea, a group of conifer trees. *Molecular Biology and Evolution* **19**, 736-747

Kuwabara C, Imai R (2009) Molecular basis of disease resistance acquired through cold acclimation in overwintering plants. *Journal of Plant Biology* **52**, 19-26

Labandeira C, LePage B, Johnson A (2001) A *Dendroctonus* bark engraving (*Coleoptera: Scolytidae*) from a middle Eocene *Larix* (Coniferales: Pinaceae): Early or delayed colonization? *American Journal of Botany* **88**, 2026-2039

Larcher W (2003) Stress due to extreme temperatures. In: Larcher W (Ed) *Physiological Plant Ecology*, Springer-Verlag, Berlin, Germany, pp 364-395

Lawrence SD, Novak NG (2006) Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle. *Biotechnology Letters* **28**, 593-599

Leinala EK, Davies PL, Jia Z (2002) Crystal structure of β-helical antifreeze protein points to a general ice binding model. *Structure* **10**, 619-627

Lippert D, Chowrira S, Ralph SG, Zhuang J, Aeschliman D, Ritland C, Ritland K, Bohlmann J (2007) Conifer defense against insects: Proteome analysis of Sitka spruce (*Picea sitchensis*) bark induced by mechanical woun-

ding or feeding by white pine weevils (*Pissodes strobi*). *Proteomics* **7**, 248-270

Liu J-J, Ekramoddoullah AKM, Yu X (2003) Differential expression of multiple PR10 proteins in western white pine following wounding, fungal infection and cold-hardening. *Physiologia Plantarum* **119**, 544-553

Liu J-J, Ekramoddoullah AKM, Zamani A (2005) A class IV chitinase is upregulated by fungal infection and abiotic stresses and associated with slow-canker-growth resistance to *Cronartium ribicola* in western white pine (*Pinus monticola*). *Phytopathology* **95**, 284-291

Lorenz WW, Sun F, Liang C, Kolychev D, Wang H, Zhao X, Cordonnier-Pratt M-M, Pratt LH, Dean JFD (2006) Water stress-responsive genes in loblolly pine (*Pinus taeda*) roots identified by analyses of expressed sequence tag libraries. *Tree Physiology* **26**, 1-16

Lucca AJDe, Cleveland TE, Wedge DE (2005) Plant-derived antifungal proteins and peptides. *Canadian Journal of Microbiology* **51**, 1001-1014

Malinowski R, Filipecki M (2002) The role of cell wall in plant embryogenesis. *Cellular and Molecular Biology Letters* **7**, 1137-1151

Mason ME, Davis JM (1997) Defense response in slash pine: Chitosan treatment alters the abundance of specific mRNAs. *Molecular Plant-Microbe Interactions* **10**, 135-137

Matheus N, Ekramoddoullah AKM, Lee SP (2003) Isolation of high quality RNA from white spruce tissue using a three stage purification method and cDNA cloning of a PR-10 gene. *Phytochemical Analysis* **14**, 209-215

Meins FJr, Fritig B, Linthorst HJM, Mikkelsen JD, Neuhaus J-M, Ryals J (1994) Plant chitinase genes. *Plant Molecular Biology Reports* **12**, S22-S28

Melchers LS, Apotherker-de Groot M, van der Knaap JA, Ponstein AS, Sela-Buurlage MB, Bol JF, Cornelissen BJC, van den Elzen PJM, Linthorst HJM (1994) A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. *The Plant Journal* **5**, 469-480

Merino ST, Cherry J (2007) Progress and challenges in enzyme development for biomass utilization. *Advances in Biochemical Engineering/Biotechnology* **108**, 95-120

Mitsunaga T, Iwase M, Yuki D, Koga D (2004) Intracellular localization of a class IV chitinase from yam. *Bioscience, Biotechnology, and Biochemistry* **68**, 1518-1524

Nagy N, Fossdal CG, Dalen LS, Lönneborg A, Heldal I, Johnsen Ø (2004) Effects of *Rhizoctonia* infection and drought on peroxidase and chitinase activity in Norway spruce (*Picea abies*). *Physiologia Plantarum* **120**, 1-9

Nakamura T, Ishikawa M, Nakatani H, Oda A (2008) Characterization of cold-responsive extracellular chitinase in bromegrass cell cultures and its relationship to antifreeze activity. *Plant Physiology* **147**, 391-401

Neuhaus J-M, Fritig B, Linthorst HJM, Meins FJ, Mikkelsen JD, Ryals JD (1996) A revised nomenclature for chitinase genes. *Plant Molecular Biology Reporter* **14**, 102-104

Neuhaus JM, Rogers JC (1998) Sorting of proteins to vacuoles in plant cells. *Plant Molecular Biology* **38**, 127-144

Niemelä P, Chapin FS, Danell K, Bryant JP (2001) Herbivory-mediated responses of selected boreal forests to climatic change. *Climate Change* **48**, 427-240

Nsolomo VR, Woodward S (2007) Glucanohydrolase enzyme activity in embryos of Scots, Corsican and lodgepole pines infected *in vitro* with *Heterobasidion annosum*. *European Journal of Forest Pathology* **24**, 144-153

Ohme-Takagi M, Meins F, Shinshi H (1998) A tobacco gene encoding a novel basic class II chitinase: A putative ancestor of basic class I and acidic class II chitinase genes. *Molecular and General Genetics* **259**, 511-515

Passarinho PA, van Hengel AJ, Fransz PF, de Vries SC (2001) Expression pattern of the *Arabidopsis thaliana AtEP3/AtchitIV* endochitinase gene. *Planta* **212**, 556-567

Patil RS, Ghormade VV, Deshpande MV (2000) Chitinolytic enzymes: An exploration. *Enzyme and Microbial Technology* **26**, 473-483

Pirttilä AM, Laukkanen H, Hohtola A (2002) Chitinase production in pine callus (*Pinus sylvestris* L.): A defence reaction against endophytes? *Planta* **214**, 848-852

Popp MP, Lesney MS, Davis JM (1997) Defense responses elicited in pine cell suspension cultures. *Plant Cell, Tissue and Organ Culture* **47**, 199-205

Punja ZK, Zhang Y-Y (1993) Plant chitinases and their roles in resistance to fungal disease. *Journal of Nematology* **25**, 526-540

Quecine MC, Araujo WL, Marcon J, Gai CS, Azevedo JL, Pizzirani-Kleiner AA (2008) Chitinolytic activity of endophytic *Streptomyces* and potential for biocontrol. *Letters in Applied Microbiology* **47**, 486-491

Ralph SG, Yueh H, Friedmann M, Aeschliman D, Zeznik JA, Nelson CC, Butterfield YSN, Kirkpatrick R, Liu J, Jones SJM, Marra MA, Douglas CJ, Ritland K, Bohlmann J (2006) Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant, Cell and Environment* **29**, 1545-1570

Robinson RM, Sturrock RN, Davidson JJ, Ekramoddoullah AKM, Morrison DJ (2000) Detection of chitinase-like protein in the roots of Douglas-fir trees infected with *Armillaria ostoyae* and *Phellinus weirii*. *Tree Physiology* **20**, 493-502

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

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

Sánchez-Monge R, Blanco C, Perales AD, Collada C, Carrillo T, Aragoncillo C, Salcedo G (2000) Class I chitinases, the panallergens responsible for the latex-fruit syndrome, are induced by ethylene treatment and inactivated by heating. *Journal of Allergy and Clinical Immunology* **106**, 190-195

Sathyan P (2004). Identification of drought responsive genes in aleppo pine (*Pinus halepensis*) and loblolly pine (*Pinus taeda* L). MSc thesis, Molecular and Environmental Plant Sciences, School of Graduate Studies, Texas A&M University, TX, USA, 67 pp

Selitrennikoff CP (2001) Antifungal proteins. *Applied and Environmental Microbiology* **67**, 2883-2894

Sharma P, Børja D, Stougaard P, Lönneborg A (1993) PR-proteins accumulating in spruce roots infected with a pathogenic *Pythium* sp. isolate include chitinases, chitosanases and β-1,3-glucanases. *Physiological and Molecular Plant Pathology* **43**, 57-67

Shin H, Lee H, Woo K-S, Noh E-W, Koo Y-B, Lee K-J (2009) Identification of genes upregulated by pinewood nematode inoculation in Japanese red pine. *Tree Physiology* **29**, 411-421

Shinshi H, Neuhaus J-M, Ryals J, Meins F Jr. (1990) Structure of tobacco endochitinase gene: Evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. *Plant Molecular Biology* **14**, 357-368

Singh A, Kirubakaran SI, Sakthivel N (2007) Heterologus expression of new antifungal chitinase from wheat. *Protein Expression and Purification* **56**, 100-109

Staehelin C, Granado J, Müller J, Wiemken A, Mellor RB, Felix G, Regenass M, Broughton WJ, Boller T (1994) Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases. *Proceedings of the National Academy of Sciences USA* **91**, 2196-2200

Staehelin C, Schultze M, Kondorosi E, Kondorosi A (1995) Lipo-chitooligosaccharide nodulation signals from *Rhizobium meliloti* induce their rapid degradation by the host plant alfalfa. *Plant Physiology* **108**, 1607-1614

Stefani FOP, Tanguay P, Pelletier G, Piché Y, Hamelin RC (2010) Impact of endochitinase-transformed white spruce on soil fungal biomass and ectendomycorrhizal symbiosis. *Applied and Environmental Microbiology* **76**, 2607-2614

Sturrock RN, Islam MA, Ekramoddoullah AKM (2007) Host–pathogen interactions in Douglas-fir seedlings infected by the laminated root rot fungus *Phellinus sulphurascens*. *Phytopathology* **97**, 1406-1414

Sturrock RN, Zeglen S, Turner J (2006) Laminated root rot forest health stand establishment decision aid. *BC Journal of Ecosystems and Management* **7**, 41-43

Taira T, Hayashi H, Tajiri Y, Onaga S, Uechi G, Iwasaki H, Ohnuma T, Fukamizo T (2009) A plant class V chitinase from a cycad (*Cycas revoluta*): Biochemical characterization, cDNA isolation, and posttranslational modification. *Glycobiology* **19**, 1452-1461

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599

Tateishi Y, Umemura Y, Esaka M (2001) A basic class I chitinase expression in winged bean is up-regulated by osmotic stress. *Bioscience, Biotechnology, and Biochemistry* **65**, 1663-1668

Ubhayasekera W, Rawat R, Ho SWT, Wiweger M, Von Arnold S, Chye M–L, Mowbray SL (2009) The first crystal structures of a family 19 class IV chitinase: The enzyme from Norway spruce. *Plant Molecular Biology* **71**, 277-289

Uzal EN, Gómez-Ros LV, Hernádez JA, Pedreño MA, Cuello J, Barceló AR (2009) Analysis of the soluble cell wall proteome of gymnosperms. *Journal of Plant Physiology* **166**, 831-843

Vaaje-Kolstad G, Horn SJ, van Aalten DMF, Synstad B, Eijsink VGH (2005) The non-catalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. *The Journal of Biological Chemistry* **280**, 28492-28497

van Aalten DM, Komander D, Synstad B, Gaseidnes S, Peter MG, Eijsink VG (2001) Structural insights into the catalytic mechanism of a family 18 exo-chitinase. *Proceedings of the National Academy of Sciences USA* **98**, 8979-8984

van Hengel AJ, Guzzo F, van Kammen A, de Vries SC (1998) Expression pattern of the carrot *EP3* endochitinase genes in suspension cultures and in developing seeds. *Plant Physiology* **117**, 43-53

van Hengel AJ, Tadesse Z, Immerzeel P, Schols H, Van Kammen A, De Vries SC (2001) *N*-acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. *Plant Physiology* **125**, 1880-1890

von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* **69**, 233-249

Watanabe T, Kanai R, Kawase T, Tanabe T, Mitsutomi M, Sakuda S, Miyashita K (1999) Family 19 chitinases of *Streptomyces* species: Characteriza-

tion and distribution. *Microbiology* **145**, 3353-3363

Wessels JGH (1994) Developmental regulation of fungal cell wall formation. *Annual Review of Phytopathology* **32**, 413-437

Wiweger M, Farbos I, Ingouff M, Lagercrantz U, Von Arnold S (2003) Expression of Chia4-Pa chitinase genes during somatic and zygotic embryo development in Norway spruce (*Picea abies*): Similarities and differences between gymnosperm and angiosperm class IV chitinases. *Journal of Experimental Botany* **54**, 2691-2699

Wu H, Echt CS, Popp MP, Davis JM (1997) Molecular cloning structure and expression of an elicitor-inducible chitinase gene from pine trees. *Plant Molecular Biology* **33**, 979-987

Wu H, Michler CH, LaRussa L, Davis JM (1999) The pine *pschi4* promoter directs wound-induced transcription. *Plant Science* **142**, 199-207

Xiao Y-H, Li X-B, Yang X-Y, Luo M, Hou L, Guo S-H, Luo X-Y, Pei Y (2007) Cloning and characterization of a balsam pear class I chitinase gene (*Mcchit1*) and its ectopic expression enhances fungal resistance in transgenic plants. *Bioscience, Biotechnology, and Biochemistry* **71**, 1211-1219

Xu F, Fan C, He Y (2007) Chitinases in *Oryza sativa* ssp. Japonica and *Arabidopsis thaliana*. *Journal of Genetics and Genomics* **34**, 138-150

Zamani A, Sturrock RN, Ekramoddoullah AKM, Wiseman SB, Griffith M (2003) Endochitinase activity in the apoplastic fluid of *Phellinus weirii*-infected Douglas-fir and its association with over wintering and antifreeze activity. *Forest Pathology* **33**, 299-316

Zhong R, Kays SJ, Schroeder BP, Ye Z-H (2002) Mutation of a chitinase-like gene causes ectopic deposition of lignin, aberrant cell shapes, and overproduction of ethylene. *Plant Cell* **14**, 165-179