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Cloning and characterization of chitinases from interior spruce and lodgepole pine

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ABSTRACT

Chitinases have been implicated in the defence of conifers against insects and pathogens. cDNA for six chitinases were cloned from interior spruce (*Picea glauca x engelmannii*) and four from lodgepole pine (*Pinus contorta*). The cloned interior spruce chitinases were annotated class I *PgeChia1-1* and *PgeChia1-2*, class II *PgeChia2-1*, class IV *PgeChia4-1*, and class VII *PgeChia7-1* and *PgeChia7-2*; lodgepole pine chitinases were annotated class I *PcChia1-1*, class IV *PcChia4-1*, and class VII *PcChia7-1* and *PcChia7-2*. Chitinases were expressed in *Escherichia coli* with maltose-binding-protein tags and soluble proteins purified. Functional characterization demonstrated chitinolytic activity for the three class I chitinases PgeChia1-1, PgeChia1-2 and PcChia1-1. Transcript analysis established strong induction of most of the tested chitinases, including all three class I chitinases, in interior spruce and lodgepole pine in response to inoculation with bark beetle associated fungi (*Leptographium abietinum* and *Grosmannia clavigera*) and in interior spruce in response to weevil (*Pissodes strobi*) feeding. Evidence of chitinolytic activity and inducibility by fungal and insect attack support the involvement of these chitinases in conifer defense.

Introduction

Conifer trees, including species of spruce (*Picea* spp.) and pine (*Pinus* spp.), are exposed to many different species of insect pests and pathogenic fungi. Major biochemical defenses of conifers include constitutive and induced formation of terpenoids and phenolics, as well as protein based defenses such as chitinases (Kolosova and Bohlmann, 2012).

Chitinases catalyze hydrolysis of chitin, a linear polymer of β-1,4-linked *N* acetylglucosamine that is a common constituent of fungal cell walls and the peritrophic matrix of insect digestive systems. Chitinolytic activity was demonstrated for a number of angiosperm plant chitinases *in vitro* using chitin or chitin analogs as a substrate (Collinge et al., 1993; Kasprzewska, 2003; Kirubakaran and Sakthivel, 2007; Singh et al., 2007), but to the best of our knowledge, has not been reported for genes annotated as chitinases in conifers. In angiosperms, hydrolysis of chitin resulted in growth inhibition of a variety of fungal pathogens *in vitro* (Kirubakaran and Sakthivel, 2007; Li et al., 2003; Schlumbaum et al., 1986; Singh et al., 2007; Verburg and Huynh, 1991; Ye and Ng, 2005). Induction of chitinases by pathogens was also demonstrated in several angiosperms (Kasprzewska, 2003) and overexpression of selected chitinases in transgenic plants resulted in increased resistance against fungal pathogens (Jayaraj and Punja, 2007; Vellicce et al., 2006; Xiao et al., 2007).

The ability of chitinases to damage insect peritrophic matrix was demonstrated *in vitro* and *in vivo* for chitinases of entomopathogenic fungi and nematodes (Kramer and Muthukrishnan, 1997). Expression of a poplar chitinase in transgenic tomato led to slower development of Colorado potato beetle (Lawrence and Novak, 2006). In conifers, induction of chitinase expression by

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pathogen attack, insect herbivory and wounding was demonstrated for species of spruce and pine (Davis et al., 2002; Hietala et al., 2004; Kozlowski and Metraux, 1998; Liu et al., 2005; Nagy et al., 2004; Ralph et al., 2006), and increased expression of chitinases was associated with Norway spruce (*Picea abies*) resistance to the pathogenic fungus *Heterobasidion annosum* (Fossdal et al., 2006). In addition to their involvement in defense, chitinases also function in plant development. For example, chitinases may be involved in the control of embryogenesis (Kragh et al., 1996; Wiweger et al., 2003).

Chitinases are represented by large gene families in plants (Graham and Sticklen, 1994) and members vary in biochemical and biological properties. Plant chitinases are divided into seven classes (class I–VII) based on their sequence and domain structure (Meins et al., 1994; Neuhaus, 1999). Class I chitinases contain a catalytic domain and an additional cysteine-rich domain which is suggested to be involved in chitin binding. These chitinases are typically localized to the vacuole, although some are apoplastic (Graham and Sticklen, 1994). Class I chitinases are known to have high chitinolytic activity, which is not known for class II chitinases. Class II chitinases are localized extracellularly. Their catalytic domain is highly similar to that of class I chitinases. Class II chitinases lack the cysteine-rich domain (Graham and Sticklen, 1994). Class III chitinases have no obvious sequence similarity with class I and II plant chitinases but have some sequence similarity to bacterial chitinases (Graham and Sticklen, 1994). Class IV chitinases have a high level of sequence similarity with class I chitinases but have several deletions, a truncated C-terminus (Meins et al., 1994) and appear to be localized extracellularly (Graham and Sticklen, 1994). Class V chitinases have a duplicated cysteine-rich domain (Meins et al., 1994). Class VI chitinases have sequence similarity to bacterial chitinases, but no obvious sequence similarity to the class I–V chitinases (Meins et al., 1994). Class VII chitinases are highly similar to class IV chitinases but lack the chitin binding domain (Neuhaus, 1999).

Previously cloned chitinases in conifers include class I, II and IV chitinases (Davis et al., 2002; Hietala et al., 2004; Liu et al., 2005; Wiweger et al., 2003; Wu et al., 1997). Involvement of many of these chitinases in conifer defense was proposed based on their induction by wounding, fungal inoculation, or elicitor treatment (Davis et al., 2002; Hietala et al., 2004; Kozlowski and Metraux, 1998; Liu et al., 2005; Nagy et al., 2004; Ralph et al., 2006). To our knowledge, there are no reports on the functional characterization of conifer chitinases. Here, we report the cloning of cDNAs encoding six different chitinases from interior spruce (*Picea engelmannii x glauca*) representing classes I, II, IV and VII and four different chitinases from lodgepole pine (*Pinus contorta*) representing classes I, IV and VII. For this stydy chitinolytic activity is demonstrated for three class I chitinases. Gene specific transcript analysis showed induction of most of the tested chitinases by fungal inoculation and wounding in interior spruce and lodgepole pine and by weevil feeding in interior spruce.

Results

Classification of cloned interior spruce and lodgepole pine chitinases

Using published (Hall et al., 2013; Ralph et al., 2008) and newly developed EST sequences, six different candidate chitinases were cloned from interior spruce and four different candidate chitinases from lodgepole pine as full length cDNAs. Chitinase sequences were assigned to classes based on the established classification systems (Meins et al., 1994; Neuhaus, 1999). Sequence alignment of the conifer chitinases with previously characterized chitinases (Hamel et al., 1997; Liu et al., 2005) confirmed the presence of characteristic plant chitinase domains of different classes, including the catalytic domain and hydrophobic N-terminal signal peptides present in all cloned interior spruce and lodgepole pine chitinases and the chitin binding domain and C-terminal extension present in some of them (Fig. 1, Supplemental Fig. 1). The cloned chitinases were named based on the established "Chia" nomenclature system which indicates the chitinase family, followed by a number indicating chitinase class with the final number indicating individual class members (Neuhaus, 1999).

Interior spruce chitinases *PgeChia1-1* (cDNA encoding a predicted protein of 338 amino acids), *PgeChia1-2* (341 amino acids) and lodgepole pine chitinase *PcChia1-1* (341 amino acids) contained the cysteine-rich chitin binding domain and had over 50% amino acid sequence identity with tobacco class I chitinase (GenBank Accession No. X64519.1). These three were assigned to class I (Fig. 1). Interior spruce chitinase *PgeChia2-1* (308 amino acids) had 79% amino acid sequence identity with class I chitinase *PgeChia1-1* but was lacking the cysteine-rich domain; it was assigned to class II (Fig. 1). *PgeChia2-1* represents a class chitinase. In contrast to the previously cloned class II chitinase *Pschi4* from eastern white pine (*Pinus strobus*) (Wu et al., 1997), *PgeChia2-1* does not show a deletion in the catalytic domain (Fig. 1). Class I and II cDNAs encode for predicted proteins with variable C-terminal extensions. Interior spruce *PgeChia4-1* (266 amino acids) and lodgepole pine *PcChia4-1* (274 amino acids) were assigned to class IV based on a deletion in the chitin binding domain, several deletions in the catalytic domain and lack of a C-terminal extension (Fig. 1). *PgeChia4-1* and *PcChia4-1* have, respectively, 93% and 85% amino acid sequence identity with the previously characterized Norway spruce class IV chitinase *Chia4-Pa1* (Wiweger et al., 2003). Interior spruce *PgeChia7-1* (231 amino acids) and *PgeChia7-2* (230 amino acids), as well as lodgepole pine *PcChia7-1* (231 amino acids), and *PcChia7-2* (233 amino acids) were assigned to class VII based on similarity of the catalytic domain of class IV chitinases and the absence of the cysteine-rich domain and C-terminal extension (Fig. 1).

Class I proteins have chitinolytic activity

The ten chitinases cloned from interior spruce and lodgepole pine were expressed in *Escherichia coli* using the pMAL-4X (NEB) expression vector that contains a maltose-binding-protein (MBP) tag. Initial attempts of expression of the chitinases using the His-Tag expression vector pET-28b(+) resulted in insoluble protein that was recalcitrant to functional characterization. Soluble recombinant proteins were obtained using the pMAL-4X expression system. The MBP tag supports proper folding of soluble protein (PerezMartin et al., 1997). All proteins were partially purified using amylose resin. The yield of purified proteins varied from 2 mg/L to 30 mg/L depending on the target protein. The best expressed proetins were PgeChia1-1, PgeChia1-2 and all four of the class VII chitinases. The purified proteins were used to determine the presence of chitinolytic activity.

Using CM-chitin-RBV as a substrate established the presence of chitinolytic activity for all of the tested class I chitinases: PgeChia1-1, PgeChia1-2 and PcChia1-1. Boiled chitinases and MPB tag isolated from *E. coli* culture carrying an empty vector did not exhibit chitinolytic activity. Interior spruce and lodgepole pine proteins of class II, IV and VII did not exhibit chitinolytic activity under the conditions tested. We also tested other methods for determination of possible chitinase activity, including measuring the release of the reducing end group N-acetamino-glucose from colloidal chitin using dinitrosalicylic acid (Kirubakaran and Sakthivel, 2007) and using 4-methylumbelliferyl (4MU) labelled chitin analogs such as 4-MU-$(GlcNAc)_1$ and 4-MU-$(GlcNAc)_3$ (Eilenberg et al., 2006). However, these assay systems did not detect chitinolytic activity with any of the proteins tested. Background corrected increase of