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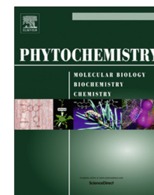

Cloning and characterization of chitinases from interior spruce and lodgepole pine

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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.
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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

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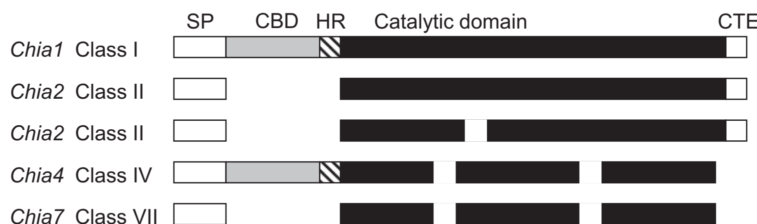

Fig. 1. Schematic structure of chitinases characterized in conifer species so far. SP – signal peptide, CBD – chitin binding domain, HR – hinge region, CTE – C terminal extension. The schematic representation was modified from Hamel et al. (1997) and extended based on Neuhaus (1999).

absorbance at 560 nm in the CM-chitin-RBV assay system results from chitinase catalyzed substrate cleavage. In this assay system, the three class I chitinases showed a similar range of linearity of activity, detected as change of absorbance plotted against amount of protein, before reaching a plateau of protein saturation (Fig. 2). Chitinolytic activity of the three chitinases was comparable to previously characterized *Aeromonas schubertii* chitinase that was assayed using a similar method (Guo et al., 2004). All three class I chitinases exhibited highest activity at pH 7 (Fig. 2).

Induced transcript levels of chitinases in interior spruce inoculated with Leptographium abietinum

Transcript levels of the two class I chitinases, *PgeChia1-1* and *PgeChia1-2*, in comparison with transcript levels of *PgeChia2-1* and *PgeChia 4-1*, were analysed in interior spruce inoculated with the spruce beetle associated blue-stain fungus *Leptographium abietinum* at 6 h, 2 days and 2 weeks after inoculation (Fig. 3A). Results from quantitative (q)RT-PCR analyses are shown as relative expression normalized to eukaryotic translation initiation factor TIF5A, and as fold induction relative to unwounded control treatment in case of the wounding treatment and relative to control and wounding treatment in case of fungal treatment (Supplemental Table 1). Changes of transcript abundance were considered statistically significant with >2-fold change (FC) and $p < 0.5$. All transcript levels were analyzed using gene-specific primers revealing inducibility for all four targets tested. All four genes showed similar patterns of induction with stronger responses to fungal inoculation compared to the response to mechanical wounding, and a peak response to fungal inoculation at 2 days post inoculation. For the two class I chitinases, PgeChia1-1 showed a particularly strong induction (wounding vs. control: 53.1 FC; fungal treatment vs. control: 189.4 FC at 2 days post treatment). Class IV *PgeChia4-1* chitinase was also strongly induced (wounding vs. control: 36.6 FC; fungal treatment vs. control: 97.7 FC at 2 days post treatment).

Induced transcript levels of chitinases in lodgepole pine inoculated with Grosmannia clavigera

Transcript levels of four chitinases were analysed in lodgepole pine inoculated with the mountain pine beetle associated

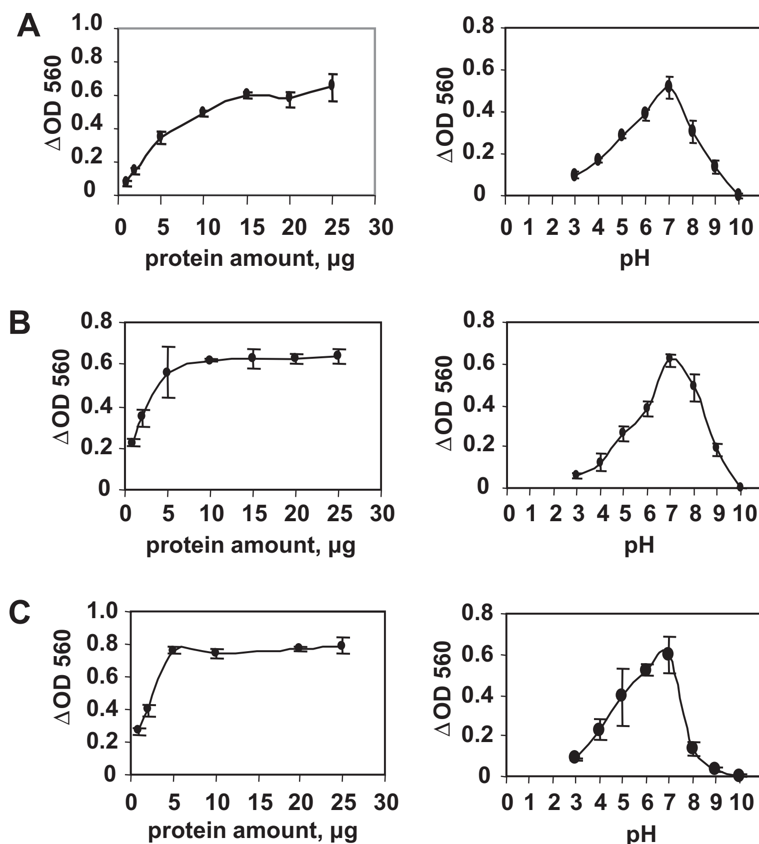

Fig. 2. Chitinolytic activity and effect of the amount of protein and pH on the activity of conifer class I chitinases. A – PgeChia1-1; B – PgeChia1-2; C – PcChia1-2. A – PgeChia1-1; B – PgeChia1-2; C – PcChia1-2. Results are shown as average of three replicates with standard deviation.

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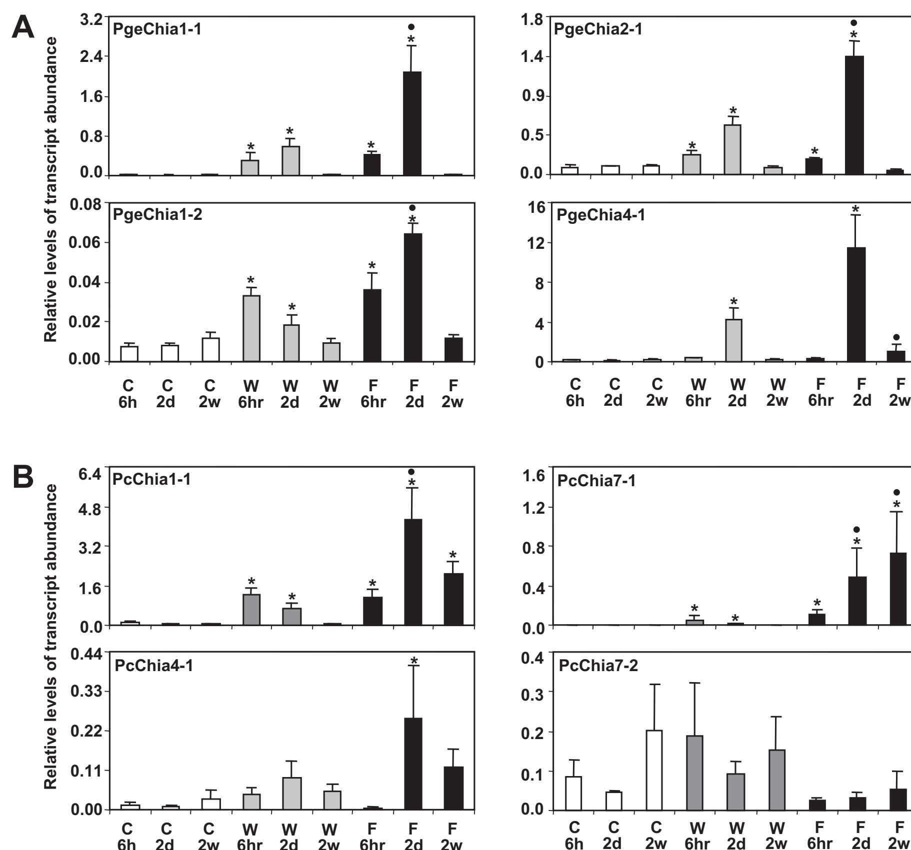

Fig. 3. qRT-PCR analysis of chitinase transcripts levels in interior spruce and lodgepole pine treated with wounding (W) or fungal inoculation (F) compared to controls (C). (A) Relative expression levels of four transcripts of chitinase family in interior spruce bark untreated, treated with wounding and inoculated with *L. abietinum* during the time course of 6 h (6 h), 2 days (2d), and 2 weeks (2w). (B) Relative expression levels of four transcripts of chitinase family in lodgepole pine bark untreated, treated with wounding and inoculated with *G. clavigera* during the time course of 6 h, 2 days, and 2 weeks. Values represent mean + standard error of the mean. Transcript abundance is shown relative to TIF5A transcript in the same sample. * – indicated statistically significant difference compared to control, and a dot indicated statistically significant difference compared to wounding.

blue-stain fungus *Grosmannia clavigera* at 6 h, 2 days and 2 weeks after inoculation (Fig. 3B, Supplemental Table 1). Class I *PcChia1-1* transcript levels showed a strong increase in response to wounding (22 FC) and fungal inoculation (144.7 FC) at 2 days post-treatment. *PcChia4-1* and *PcChia7-1* transcripts were also strongly induced by fungal inoculation at 2 days post-treatment (28.4 FC and 572.7 FC, respectively) relative to controls (Supplemental Table 1). Transcript levels of *PcChia7-1* induced by fungal treatment remained high at 2 weeks post-treatment compared to control (366.0 FC) (Fig. 3B, Supplemental Table 1). This transcript was also induced by wounding, but to lesser extent as compared to fungal treatment (wounding vs. control at 6 h showed 15.9 FC and at 2 days 13.8 FC). *PcChia7-2* transcript levels were not significantly affected by wounding or fungal inoculation (Fig. 3B, Supplemental Table 1).

Induced transcript levels of chitinases in interior spruce exposed to white pine weevil feeding

Transcript levels of *PgeChia1-1*, *PgeChia1-2*, *PgeChia2-1*, and *PgeChia4-1* showed strong induction in response to weevil feeding in interior spruce. *PgeChia1-1* was highly induced at 2 days of weevil feeding (FC 125.6) and continued to be induced but to a lesser extent at 2 weeks of treatment (FC 18.0) (Fig. 4, Supplemental Table 2). The induction *PgeChia1-2* followed a similar pattern. Transcript levels of the class IV chitinase *PgeChia4-1* also showed strong induction at 2 days of weevil feeding (FC 47.6) and continued to increase by 2 weeks of weevil feeding (FC 88.4) (Fig. 4, Supplemental Table 2). Class II PgeChia2-1 chitinase expression induction followed similar patterns as class I chitinases (Fig. 4).

Evaluation of antifungal activity

The three class I chitinases (PgeChia1-1, PgeChia1-2, PcChia1-1) did not show obvious antifungal activity against *G. clavigera* (strain SLKw1407) and *L. abietinum* (strain 2PG6P-La) when tested using three different spore germination methods, including spore germination in liquid suspension in the presence of a chitinase protein (Chen et al., 2007), germination of the spores on agar plate in the presence of a chitinase protein (Mauch et al., 1988) or germination of pre-incubated (for 24–48 h) spores with chitinase protein on agar plate. In all cases concentration of the purified chitinase protein was 2 mg/ml. MBP tag protein (2 mg/ml) was used as a control. In all of these tests, spore germination and fungal growth were not inhibited by the presence of the tested chitinases.

Discussion

The involvement of the chitinase gene family in conifer defense is suggested by a number of studies that correlated chitinase expression with the conifer defense response and disease resistance (e.g., Davis et al., 2002; Fossdal et al., 2006; Hietala et al., 2004; Kozlowski and Metraux, 1998; Liu et al., 2005; Nagy et al.,

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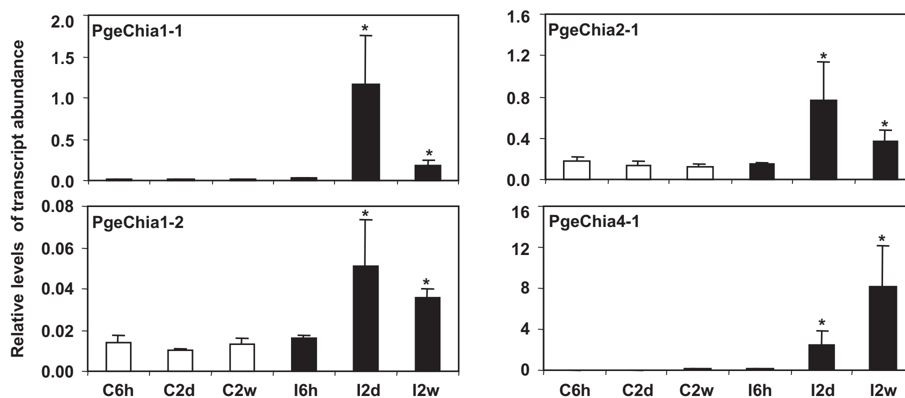

Fig. 4. qRT-PCR analysis of chitinase transcripts levels in interior spruce exposed to weevil feeding. Bars represent relative expression levels of four transcripts of the chitinase family in interior spruce bark untreated, or treated with weevil feeding during the time course of 6 h (6 h), 2 days (2 d), and 2 weeks (2 w). Values represent mean + standard error. Transcript abundance is shown relative to the TIF5A transcript in the same sample. C – control, I – weevil (insect) treatment. * – indicated statistically significant difference compared to control.

2004; Ralph et al., 2006). Here, six interior spruce chitinases and four lodgepole pine chitinases were cloned and for the first time established the presence of chitinolytic activity for three conifer class I chitinases, *PgeChia1-1*, *PgeChia1-2* and *PcChia1-1*.

The six interior spruce chitinases and four lodgepole pine chitinases represented four different classes of chitinases, class I, class II, class IV and class VII. All ten chitinases have a characteristic N-terminal signal peptide with a highly hydrophobic core that presumably targets proteins into the secretory pathway (Graham and Sticklen, 1994). The interior spruce chitinases of class I and II (*PgeChia1-1*, *PgeChia1-2* and *PgeChia2-1*) and the lodgepole pine class I chitinase *PcChia1-1* also have a C-terminal extension that may target these chitinases to vacuoles (Graham and Sticklen, 1994). It was previously demonstrated that a short (six amino acids) C-terminal sequence is necessary and sufficient for targeting of tobacco class I chitinase to the vacuole (Neuhaus et al., 1991). Only the class I and IV chitinases from interior spruce and lodgepole pine had the cysteine rich domain that has been shown to have chitin binding properties in angiosperm chitinases (Iseli et al., 1993).

Functional characterization established chitinolytic activity only in class I interior spruce (PgeChia1-1, PgeChia1-2) and lodgepole pine (PcChia1-1) chitinase proteins, consistent with previous observation in angiosperms that class I chitinases contribute much of the chitinolytic activity in plant tissues where different chitinases are present (Graham and Sticklen, 1994). Class I chitinases generally have higher chitinolytic activity than class II chitinases, which lack a known chitin binding domain, as was demonstrated for barley and tobacco chitinases perhaps supporting the importance of the chitin binding domain to catalytic activity (Graham and Sticklen, 1994). However, another study involving a direct comparison of the tobacco class I chitinases activity, with and without a chitin binding domain, demonstrated that this domain may not be necessary for catalytic activity (Iseli et al., 1993). The absence of detectable catalytic activity, using three different assay systems, in interior spruce class II chitinase *PgeChia2-1* which lacks the chitin binding domain but shares 93% sequence identity in the catalytic domain with class I chitinase *PgeChia1-1* supports the relevance of the chitin binding domain for chitinolytic activity of interior spruce chitinases.

Gene specific transcript analyses demonstrated strong induction of class I (*PgeChia1-1* and *PgeChia1-2*) and other tested interior spruce chitinases (class II *PgeChia2-1* and class IV *PgeChia4-1*) in response to *L. abietinum* fungal inoculation or weevil feeding. Similarly, the lodgepole pine class I *PcChia1-1* and class IV *PcChia4-1* chitinase were strongly induced by *G. clavigera*. *G. clavigera* (Diguistini et al., 2011) and *L. abietinum* are bark beetle vectored fungal pathogens of pine and spruce species, respectively. Strong inducibility of chitinases by both weevil and fungal treatments in interior spruce indicate a possible broader role of these chitinases in spruce defense. Induction of chitinases with antifungal properties and/or anti-insect properties may be advantageous to conifers that are often exposed to insect and fungal attack simultaneously (Huber et al., 2004; Paine et al., 1997).

In our assay systems, *in vivo* antifungal activity was not detected for the three interior spruce and lodgepole pine class I chitinases. This negative result may be attributed to the complexity of the fungal cell wall (Bowman and Free, 2006) or may be due to possible lack of solubility of the products of the tested chitinase activity in the aqueous in vivo assay system (Grover, 2012) and warrants future work using combinations of chitinases and other cell wall active enzymes, such as β-1,3-glucanase (Mauch et al., 1988).

Additional recent annotation of the chitinases available from the Sitka spruce full length cDNA collection (Ralph et al., 2008) and the hybrid white spruce (Birol et al., 2013) and Norway spruce (Nystedt et al., 2013) genome assemblies revealed at least 20 different chitinases in each species. The presence of families of chitinases in conifers is consistent with a role to cope with a diverse suite of pathogens (Bishop et al., 2000) and may also be indicative of other functions.

Conclusion

Based on full length cDNA cloning, the chitinolytic activity for class I chitinases in two conifer species, interior spruce and lodgepole pine, was demonstrated. These conifer species are of much economic and ecological importance in western Canada. Lodgepole pine is the major host in the current mountain pine beetle and *G. clavigera* epidemic.

Experimental

Plant material fungal material

Interior spruce (*Picea glauca x engelmannii*, clone I1026) were grown to three years of age outside at the University of British Columbia (UBC) under natural light and environmental conditions (Ralph et al., 2008). One week prior to treatment, plants were transferred to the UBC greenhouse with greenhouse temperature fluctuating between 20 and 24 °C and an average humidity of 45%. Three year old lodgepole pine (*Pinus contorta*), grown from

seeds collected in the interior of British Columbia, were purchased from Surrey Nursery, BC. Lodgepole pine seedlings were grown outside of the UBC green house and transferred to the UBC greenhouse one week prior to treatments.

Inoculation procedure

Interior spruce was inoculated with *L. abietinum* fungus, strain 2PG6P-La (Alamouti et al., 2007). Lodgepole pine was inoculated with *G. clavigera* (strain SLKw1407) (DiGuistini et al., 2007; Diguistini et al., 2011). Fungal inoculation was performed using a modified method of Croteau et al. (1987). Fungal mycelia were grown on malt agar covered with porous cellophane (Amersham) at 24 °C for one week and collected in the active state of fungal growth. Fungal mycelium was cut into pieces of 0.5 × 1 cm. For inoculation, trees were wounded with a sterile needle at the lower part of the stem. Four equally spaced puncture wounds (~3 mm deep and 3–4 mm wide) were introduced on two opposing sides of a 10–12 cm lower stem section (first year of growth). For each wound, fungus from a single 0.5 × 1 cm^2 film section was applied to the wound surface. Wounding treatment was identical to the type of wounding that was used in fungal inoculations with no fungus introduced. Control trees did not receive any treatment. Successful establishment of fungus in treated trees was confirmed by re-isolation of fungus. Bark tissue was collected at 6 h, 2 days, and 2 weeks of treatment.

Weevil treatment

Adult white pine weevils (*Pissodes strobi*) were collected form infested leaders of Sitka Spruce at the British Columbia Ministry of Forests research plantations at Campbell River, Vancouver Island, BC. Weevils were kept without food on moist filter paper for 48 h before they were placed on the trees. Five weevils were placed on each tree and caged on the lower stem section corresponding to the first year of growth (about 10–12 cm). Bark tissue was collected at 6 h, 2 days, and 2 weeks of treatment.

Lodgepole pine cDNA library construction and DNA sequencing

The cDNA library was prepared from bark of three year old lodgepole pine inoculated with *G. clavigera.* Bark tissue, with phloem attached, was harvested at 6 h, 2 days, and 2 weeks of treatment (four trees per time point). Total RNA was extracted using a published protocol (Kolosova et al., 2004). The same amounts of RNA from each sample were pooled for library construction using pBluescript II XR cDNA Library Construction Kit (Stratagene). Sanger EST sequencing was performed on the fungus-induced tissue cDNA library as was previously described (Ralph et al., 2008) resulting in 1,222 ESTs which represent 1030 unique transcripts.

Full length cDNA cloning

Six full length cDNAs for interior spruce chitinases sequences were identified in the EST and clone collections of the Treenomix project described in Ralph et al. (2008) and completely sequenced. The following cDNA clones, representing four different classes of chitinases, were characterized: *PgeChia1-1* was cloned based on EST WS00922_B21, *PgeChia1-2* based on EST WS00929_K15, *PgeChia2-1* based on EST WS00927_N15, *PgeChia4-1* based on EST WS00924_F07, *PgeChia7-1* based on EST WS00951_K15, *PgeChia7-2* based in EST WS00949_I04 (NCBI, Treenomix.ca). Two full length lodgepole pine chitinases sequences were identified in the lodgepole pine EST sequences developed as part of the present project. These chitinases represent class VII: *PcChia7-1* was cloned based on EST WS0353_A23 (GenBank ID: GW725974.1) and *PcChia7-2* based in EST WS0352_P07 (GenBank ID: GW725947.1). Two additional lodgepole pine chitinases *PcChia1-1* and *PcChia4-1* were cloned using lodgepole pine partial EST sequences of WS0354_C23 and WS0354_D22, respectively. Full length cDNA for these latter two ESTs was obtained by PCR amplification of the coding region. A 5′ end degenerate primer for *PcChia1-1* (5′ GCTGAKATAGAAACGAGTGTCTGN) was designed just upstream of the coding region based on the alignment of orthologous full length EST sequences available from loblolly pine (TA991_3352, TA1024_3352). Similarly, a 5′ primer for *PcChia4-1* (5′ GSGGARTCT-TAAGRAATTATTSGN) was designed using Sitka spruce (WS02717_M22) and loblolly pine (TA795_3352, TA906_3352) ESTs. The 3′ primers for these clones were designed based on available EST sequences. PCR products of 1 kb (*PcChia1-1*) and 0.8 kb (*PcChia4-1*) were amplified using fungus induced lodgepole pine cDNA. These PCR products were initially subcloned into pJET1.2 vector using CloneJET PCR Cloning Kit (Fermentas) for sequence analysis.

The six interior spruce and four full length lodgepole pine chitinase full length cDNAs were subcloned into *Hind*III and *Bam*HI restriction cites of the pMal-p4X expression vector (New England Biolabs, Pickering, Ontario).

Heterologous expression and purification of chitinases

E. coli BL21(DE3) competent cells were transformed with the pMal-p4X cDNA constructs. Cells were grown at 37 °C and 250 rpm in LB medium containing 100 μg/ml of ampicillin and 2 g/L glucose to an OD_{600} of 0.5, at which point 0.3 mM IPTG was added and the cultures were incubated for another 16–20 h. Cells were collected by centrifugation and chitinase protein was extracted with buffer containing 20 mM Tris–HCl pH7.4, 200 mM NaCl, 1 mM EDTA, 40 μg/ml DNAse (Sigma, Oakville, ON, Canada) and 40 μg/ml RNAse (Sigma) and protease inhibitor coctail [50 μM TPCK (chymotrypsin-like), 50 μM TLCK (trypsin-like), 2 μM Leupeptin (serine, cysteine), 1 μM E64 (cysteine), 1 μM Pepstatin (aspartic), 10 mM 1,10-phenanthroline (mettaloproteases), 0.5 mM PMSF (serine), 0.5 mM AEBSF (serine)].

Recombinant proteins were partially purified using amylose resin (NEB) following the pMal-p4X expression and purification manufacturer's instructions. Briefly, cells were sonicated and cell debris spun down, then supernatant was applied to the amylose column. After washing with 12 column volumes of buffer containing 20 mM Tris–HCl pH7.4, 200 mM NaCl, 1 mM EDTA, protein was eluted with buffer containing 20 mM Tris–HCl pH7.4, 200 mM NaCl, 1 mM EDTA and 10 mM maltose. The size of purified proteins was confirmed using SDS–PAGE.

Enzyme assays

Chitinolytic activity was assayed using a modified protocol (Ramirez et al., 2004). Four to 25 μg of protein in 30 μl of 0.1 M Tris–HCl pH7 was combined with 90 μl of carboxymethyl Remazol Brilliant Violet-Chitin (2 mg/ml) (CM-Chitin-RBV, Loewe Biochemica, Munich, Germany). The resulting assay mixture was incubated at 50 °C for 30 min, then reactions were stopped by adding 10 μl of 1 M HCl (to adjust to pH2), CM-Chitin-RBV that was not digested was precipitated on ice for 10 min and then samples were centrifuged for 5 min at 6000 g. Note, 50 °C was the optimal temperature for the enzyme essay based on the evaluation of a temperature range from 20 to 60 °C for all chitinases that exhibited activity, consistent with previous reports for angiosperm chitinases (Kirubakaran and Sakthivel, 2007; Li et al., 2003). The supernatant absorbance was measured at 560 nm using a Spectra Fluor Plus plate reader (Tecan, San Jose, CA). Three independent measurements were performed per each of three sample replicates.

Samples at 0 min of reaction time were used as controls and their absorption was subtracted from the absorption of the assay samples to evaluate the amount of released chitin. A change of absorbance (Δ560 nm) of 0.1 corresponded to 13 µg of released RBV chitin. Protein isolated from IPTG induced cells containing empty plasmid did not show detectable chitinolytic activity.

The effect of pH on the enzymatic activity of the purified chitinases was assessed within a pH range of 3–10 using 0.1 M sodium acetate buffer (pH 3.0, 4.0, 5.0), 0.1 M phosphate buffer (pH 6.0), 0.1 M Tris–HCl buffer (pH 7.0, 8.0) and 0.1 M Glycine-NaOH buffer (pH 9.0, 10.0).

qRT-PCR analysis of transcript levels

Interior spruce and lodgepole pine bark tissue from the area of fungal inoculation or weevil feeding and from corresponding section of control trees was harvested directly into liquid nitrogen 6 h, 2 days, 2 weeks post-treatment. Tissue was stored at −80 °C. Three biological replicates were used per time point and treatment for interior spruce inoculated with fungus; four biological replicates were used per time point and treatment for lodgepole pine inoculated with fungus and four biological replicates were used for interior spruce subjected to weevil feeding for each time point. Total RNA was extracted following a previously reported protocol (Kolosova et al., 2004). The qRT-PCR analysis was performed as previously described (Ralph et al., 2007). Gene specific primers (Supplemental Table 3) were designed using previously described criteria (Ralph et al., 2006). A minimum of three biological replicates were used for each quantification and each cDNA template. Transcript abundance was normalized to translation initiation factor 5A (TIF5A, IS0013_F24, GenBank ID: DR448953). For statistical analysis a linear model was fit (treating each condition at each time point as a factor) for each gene using data from three biological replicates for each treatment at three time points (6 h, 2 days and 2 weeks). The pairwise differences between conditions at three time points were estimated and statistical significance was assessed using t statistics.

Nucleotide sequence accession numbers

All ESTs developed and used in this project are available in NCBI GenBank. The nucleotide sequences of the six interior spruce chitinases and four lodgepole pine chitinases were deposited into NCBI GenBank with the following accession numbers: GenBank ID: HM219843 (*PgeChia1-1*), GenBank ID: HM219844 (*PgeChia1-2*), GenBank ID: HM219845 (*PgeChia2-1*), GenBank ID: HM219846 (*PgeChia4-1*), GenBank ID: HM219847 (*PgeChia7-1*), GenBank ID: HM219848 (*PgeChia7-2*), GenBank ID: HM219849 (*PcChia1-1*), GenBank ID: HM219850 (*PcChia4-1*), GenBank ID: HM219851 (*PcChia7-1*), GenBank ID: HM219852 (*PcChia7-2*).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.02.006.

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