Diverse chitinases are invoked during the activity-dormancy transition in spruce

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Abstract North temperate tree species such as white spruce (*Picea glauca* [Moench] Voss) have evolved strategies to protect themselves against abiotic and biotic stresses that trees encounter during the inclement winter months. Chitinases not only play well-documented roles in plant defense but also function during physiological and developmental preparations for overwintering, including growth cessation, cold and desiccation acclimation, and dormancy acquisition. Phylogenetic analysis of 31 white spruce and 52 Norway spruce chitinases identified genes falling into each of the five clusters, which sometimes—but not always—separated the different biochemical classes of chitinases. Digital expression profiling of white spruce and Norway spruce chitinases across multiple conditions revealed a range of spatiotemporal expression patterns. Transcript abundance profiling in buds, needles, stems, and roots by quantitative RT-PCR suggested roles for eight white spruce chitinases during the growth-to-dormancy transition. In silico analyses of these eight sequences suggested that two cluster 2/class I chitinases function as chitinolytic enzymes in the tree's constitutive defense arsenal during the winter months. A cluster 2/class I, cluster 2/class II, and cluster 1/class IV chitinase each exhibit hallmarks of antifreeze proteins. Additionally, two cluster 2/class I chitinases and a cluster 1/

class IV chitinase may serve as vegetative storage proteins. One cluster 3/class II chitinase exhibited attributes suggesting that it is a chitinase-like gene functioning in cell wall synthesis. Taken together, our results imply that dormancy-associated chitinases act in concert to (1) confer protection against freezing injury, pests, and pathogens, (2) store nitrogen, and (3) promote cell maturation that precedes growth cessation.

Keywords Defense · Antifreeze proteins · Cell wall · Phylogenetic analysis · Quantitative RT-PCR · White spruce · Norway spruce · *Picea glauca* · *Picea abies*

Introduction

North temperate forest trees exhibit a number of adaptations that enable these long-lived species to withstand the inclement conditions of winter. These adaptive traits include dormancy acquisition, bud development and the concomitant cessation of growth, as well as acquisition of cold and desiccation tolerance, seasonal nitrogen remobilization, and, in deciduous species, leaf senescence (Cooke et al. 2012). Several genomic studies over the last decade have revealed the large-scale changes that occur to the transcriptome of shoot tips, needles, and stems during the transition from active growth to dormancy of both coniferous and angiosperm forest trees such as *Populus* spp. (Schrader et al. 2004; Druart et al. 2007; Ruttink et al. 2007; Park et al. 2008; Karlberg et al. 2010; Resman et al. 2010), *Pinus* spp. (Joosen et al. 2006), and *Picea* spp. (Holliday et al. 2008; Asante et al. 2011), including white spruce (*Picea glauca* [Moench] Voss; El Kayal et al. 2011; Galindo-González et al. 2012). Several of these studies have highlighted the large number of stress-associated genes that are upregulated during the transition from active growth to dormancy, such as genes coding for dehydrins, thaumatins,

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chaperonins, chitinases, glyoxylases, and oxidative stress responses (e.g., Druart et al. 2007; Holliday et al. 2008; Galindo-González et al. 2012). Galindo-González et al. (2012) further demonstrated that a subset of these proteins figure prominently in proteomic profiles of white spruce stems during the transition to dormancy. These authors observed that protein levels of multiple chitinases became particularly abundant in white spruce prior to dormancy acquisition, suggesting that chitinases play roles in biochemical, cellular, and/or physiological processes that are important for tree survival during autumnal acclimation and the ensuing winter season.

Roles for chitinases in plant defense are well-known. Chitinases from many species are strongly induced in plants challenged with fungal or bacterial pathogens (e.g., Lawton et al. 1992; Collinge et al. 1993; Sela-Buurlage et al. 1993; Melchers et al. 1994; Mason and Davis 1997; Wu et al. 1997; Salzer et al. 1997; Büchter et al. 1997; Hong et al. 2000; Hietala et al. 2004). Several chitinases are classified as pathogenesis-related (PR) proteins, making up the PR-3, PR-4, PR-8, and PR-11 groups of these defense-associated proteins (van Loon et al. 2006). Many chitinases have been shown to hydrolyze chitin and effectively inhibit tip growth of fungal hyphae through breakdown of this fungal cell wall component. Some chitinases also exhibit lysozyme-like activity and are active against the peptidoglycan component of bacterial cell walls (Collinge et al. 1993).

Chitinases comprise a large and diverse gene family, however, and characterization of other chitinases from several species has revealed that these proteins can assume functionalities in the plant other than in defense. Chitinases have been implicated in a variety of developmental processes like seed germination (Wu et al. 2001), senescence (Hanfrey et al. 1996), and morphogenesis (Zhang et al. 2004), including somatic embryogenesis (Wiweger et al. 2003; Johnsen et al. 2005). There is increasing evidence that chitinases can mediate developmental processes through biosynthesis, assembly, and/or remodelling of various cell wall components (van Hengel et al. 2001; Zhang et al. 2004; Sánchez-Rodríguez et al. 2012; Wu et al. 2012). Chitinases can also act as antifreeze proteins. Some of the first studies recognizing the potential of chitinases to adsorb to ice-forming surfaces in cold acclimation processes were carried out in winter rye and other cereals (Hon et al. 1995; Griffith et al. 1997; Yeh et al. 2000; Pihakaski-Maunsbach et al. 2001). Chitinases with antifreeze activity have been isolated from apoplastic spaces of needles from overwintering *Pseudotsuga menziesii* (Mirb.) Franco, *Picea abies* (L.) Karst., and *Picea pungens* Engelm., indicating that chitinases play a role in cold hardiness of conifers (Zamani et al. 2003; Jarzabek et al. 2009). Finally, chitinases have also been recognized as vegetative storage proteins (Clendennen et al. 1998; Peumans et al. 2002; Meuriot et al. 2004).

Different chitinase functions are reflected in the primary and secondary structure of the protein. Chitinases are typified

by the presence of a glycosyl hydrolase domain which corresponds to the catalytic domain. The chitinase family can be subdivided into two main groups—glycoside hydrolase family 18 (GH 18) and glycoside hydrolase family 19 (GH 19)—based on differences in this domain (Davies and Henrissat 1995; Iseli et al. 1996; Li and Greene 2010). Biochemically, chitinases are grouped into classes I to VII, based on their primary structure and the presence of one or more diagnostic motifs (Collinge et al. 1993; Neuhaus 1999; Grover 2012). More recently, phylogenetic analyses of *Arabidopsis* and rice chitinases have led to designation of six clusters (Xu et al. 2007). The groupings of chitinases within these phylogenetic clusters do not mirror those of the classical biochemical classification scheme, although there are some commonalities shared between the phylogenetic and biochemical classifications (Xu et al. 2007). With the extensive characterization of many plant chitinases at the gene and protein level that has been carried out to date, in silico analyses of chitinases from other species can reveal considerable insight into their potential functions in planta.

While the accumulation of chitinases in temperate forest tree species—particularly conifers—during preparation for overwintering is well-documented (Ekramoddoullah et al. 1995, 2000; Zamani et al. 2003; Jarzabek et al. 2009), the roles that these chitinases play in the tree during this phase of the annual cycle are less clear. The objective of the present study was to analyze the chitinase gene family in white spruce using both phylogenetic and in silico characterization approaches, with a focus on chitinases that we had shown in previous studies to be differentially expressed during the transition from active growth to dormancy in this north temperate conifer species (El Kayal et al. 2011; Galindo-González et al. 2012). Given that the differentially expressed chitinases belonged to multiple classes, we hypothesized that these proteins assume different functions during the activity-dormancy transition of white spruce. Using available white spruce-expressed gene sequence resources (Rigault et al. 2011), we performed a phylogenetic and structural classification of available white spruce chitinase sequences. We then carried out in silico characterization of dormancy-associated chitinases, combined with qRT-PCR transcript profiling of these chitinases during the short day-induced activity-dormancy transition in white spruce seedlings to gain further insight into the function of these chitinases in tree overwintering.

Materials and methods

Chitinase in silico analyses

White spruce chitinase sequences identified as differentially expressed during the activity-dormancy transition (Galindo-González et al. 2012) as well as chitinases from rice,

Arabidopsis, and additional selected plant species (Xu et al. 2007) were used to identify white spruce chitinase transcript sequences representative of unigenes in the white spruce gene catalog (v3.3; Rigault et al. 2011; <ftp.gydl.com/pub/arborea>), using BLAT (Kent 2002). White spruce cDNA clones representing each of these transcripts were fully sequenced to provide confirmation of the sequence obtained from the EST assembly. Nucleotide sequences, deduced amino acid sequences, unique identifiers, and corresponding NCBI gi numbers are given in Online Resource 1. Norway spruce putative chitinase peptide sequences corresponding to CDS were identified using the same approach from ConGenIE (www.congenie.org), representing the draft Norway spruce genome sequence (Nystedt et al. 2013), and are also included in Online Resource 1. The deduced amino acid sequences were aligned using ClustalW in MEGA 5.1 (Tamura et al. 2011) with sequences from *Arabidopsis* and selected plant species representing the different chitinase classes found in plants. The parameters used included a gap opening penalty of 10 and a gap extension penalty of 0.1 for the pairwise alignments and a gap opening penalty of 10 and a gap extension penalty of 0.2 for the multiple alignment; the protein weight matrix used was Gonnet; residue-specific and hydrophilic penalties were turned; gap separation distance was set to 4; and the delay for divergence cutoff was set to 30 %. After a preliminary alignment, poorly aligned sequences and sequences covering less than 50 % of the average sequence length were discarded, and a final alignment was built using default parameters. A neighbor-joining tree was built using MEGA 5.1 under the *Poisson* model, pairwise deletion, and 1,000 bootstrap repetitions (Tamura et al. 2011). An additional tree was built only with white spruce chitinases.

Functional domains were identified using all the algorithms implemented by InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>—accessed February 17, 2015), and matching domain coordinates were translated into each of the white spruce chitinase sequences using VectorNTI® (Invitrogen, Carlsbad, CA, USA). Chitinases were aligned to a class I chitinase from tobacco (Neuhaus et al. 1991) which has most of the structural domains documented in the chitinase gene family, to resolve the presence of canonical domains and critical amino acids.

3D models were built using the SWISS-MODEL homology modeling tool under default parameters (Arnold et al. 2006) for the white spruce chitinases used in the phylogenetic analysis. Generated models were visualized using Raswin-Rasmol (Sayle and Milner-White 1995). To obtain root mean square deviation values (RMSDs), all predicted structures were compared to each other using the FAST algorithm (Zhu and Weng 2005) implemented in the ProCKSI-Server (Barthel et al. 2007; <http://www.procksi.net>—accessed February 12, 2013) for multiple comparisons. The RMSD distance matrix of all pairwise comparisons was standardized, and the values

were used to create clusters and a tree topology. The tree was visualized using ProCKSI_Viz (<http://www.procksi.net>), and the Newick tree file was exported to MEGA 5.1 (Tamura et al. 2011) to modify the tree for publication. Each of the white spruce chitinase sequences was also analyzed for intrinsically disordered regions (IDRs) using PONDR-FIT (Xue et al. 2010).

Prediction of ice-binding surfaces was done using AFPredictor (Doxey et al. 2006) (<http://doxey.uwaterloo.ca/cgi-bin/AFPredictor.cgi>—accessed February 18, 2013) with a solvent accessibility (SAS) threshold (t)=15, maximum length of allowed vectors (r)=7.55 Å, minimum length of allowed vectors (q)=4.5 Å, vector matching cutoff (v)=1.09 Å, distance threshold for clustering ordered surface carbons (OSCs) (c)=7.66 Å, polar atom distance cutoff (p)=4 Å, and SAS threshold for polar atoms=0 Å².

Putative active site residues in protein models were predicted based on structural alignments to the top five enzyme homologs using COFACTOR (Roy et al. 2012) (<http://zhanglab.ccmb.med.umich.edu/COFACTOR/>—accessed March 4, 2013). When an amino acid was predicted in the spruce protein as constituting part of the active site in at least four of the five protein alignments, it was designated a putative active site residue.

Plant material

Experiments with 2-year-old white spruce seedlings derived from Quebec provenances were conducted as described in El Kayal et al. (2011) and Galindo-González et al. (2012). Following bud burst, seedlings were grown in a complete randomized block design in controlled environment growth chambers under long days (LD; 16 h day/8 h night, 20 °C, 50 to 60 % relative humidity (RH)) for ca. 8 weeks. Shortly before seedlings were to begin bud formation, the photoperiod was changed to short-day conditions (SD; 8 h day/16 h night, 20 °C, 50 to 60 % RH) to induce synchronous bud formation. Developing buds, needles, lignified stems representing current year growth, and roots were sampled at 0, 3, 7, 14, and 70 days SD and flash frozen in liquid nitrogen. Remaining plants were maintained in SD for additional 8–15 weeks and then transferred to low temperatures (LT, 2–4 °C) for 3 to 4 weeks with continuing SD prior to harvest of buds, needles, and lignified whole stems.

RNA extraction and quantitative RT-PCR

RNA was extracted according to Pavy et al. (2008) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Primer design, cDNA synthesis, and qRT-PCR using SYBR Green were carried out as previously described (El Kayal et al. 2011). *Translation initiation factor 5A* (*TIF5A*, GenBank

DR448953) was used as the reference gene, since this gene has been used successfully in previous studies for identical material (El Kayal et al. 2011; Galindo-González et al. 2012), and did not show statistically significant differences in transcript abundance in tissues over the time course ($P=0.305$ for shoot tips, $P=0.247$ for stems, $P=0.361$ for needles, and $P=0.967$ for roots). Use of a single reference gene with empirically demonstrated invariant expression ensured that the reference could be assayed with the target gene on each and every plate, thereby reducing experimental variability. Although actin and α -tubulin are commonly used as reference genes, our previous studies showed that genes encoding both actin and α -tubulin were unsuitable, as transcript abundance varied over the time course of growth cessation (Galindo-González et al. 2012; data not shown). Gene-specific primers for the target and reference genes are included in (Online Resource 2). Four biological replicates, each with three technical replicates, were analyzed per sample. Reactions were carried out with 20 ng of cDNA in a volume of 10 μ L on an ABI PRISM 7900HT Sequence Detection System (Life Technologies, Burlington, ON). Quantification of both target and reference genes was carried out using standard curves.

Statistical analyses were performed in SAS v9.1 (SAS Institute, Cary, NC). To meet assumptions of normality and heterogeneity of variance, data were $\log_{10}$ transformed prior to analysis using general linear models (PROC GLM). Significant differences were determined by Tukey's HSD test with overall $\alpha=0.05$. In two instances, data could not be successfully transformed to meet the assumptions of GLM. In these instances, non-parametric analyses were carried out using kernel density estimation with Gaussian density as the kernel (PROC KDE), followed by pairwise comparisons by t test.

Results

Spruce chitinase genes, phylogenetic relationships, and predicted protein structures

Based on sequence similarity and the presence of one or more characteristic motifs (described in more detail below), 32 expressed sequences encoding partial or full-length putative chitinases were identified in the white spruce gene catalog that comprises more than 27,000 unique expressed genes reported by Rigault et al. (2011) and Online Resources 1 and 3. Seventeen of these have a corresponding partial or full-length genomic DNA sequence (Online Resource 1) obtained by sequence capture of PG-29 (Sena et al. 2014), the genotype used for the draft genome sequence (Birol et al. 2013). Using the same criteria, 80 partial or full-length gene models were identified in the *P. abies* 1.0 assembly (Nystedt et al. 2013).

One and four additional distinct chitinase sequences were identified in NCBI for white spruce and Norway spruce, respectively.

After filtering sequences that were too short for alignment, deduced amino acid sequences corresponding to 31 white spruce and 52 Norway spruce sequences were used to construct a phylogenetic tree, together with 58 previously characterized chitinases of *Arabidopsis thaliana* and other selected plant species (Fig. 1). The neighbor-joining tree with 1,000 bootstrap replicates yielded five well-supported clusters. A clear division between GH families 18 and 19 could be seen (Davies and Henrissat 1995; Iseli et al. 1996). The GH 19 family comprised clusters 1 through 3, with these chitinases representing structural classes I, II, IV, and VII. The GH 18 family contained clusters 4 and 5 and comprised members of chitinase classes III and V, respectively. The spruce putative chitinase sequences were distributed among each of the five clusters, with 22 white spruce and 37 Norway spruce sequences found in clusters 1, 2, and 3 of GH 19. While some clusters clearly distinguished just one type of structural class of chitinases, others represented more than one class (Fig. 1). Two subclusters of conifer chitinases could be readily distinguished within clusters 1 and 2. The topology of the tree allowed for identification of several putative orthologous pairs of white spruce–Norway spruce chitinase sequences (Online Resource 3).

In silico domain analyses were carried out for both white spruce and Norway spruce chitinase sequences (Online Resource 3). These analyses revealed that each of the 31 white spruce and 80 Norway spruce sequences contained a catalytic domain, while 23 white spruce sequences and 40 Norway spruce sequences had a predicted signal peptide. It should be noted that the seven white spruce sequences missing the signal peptide exhibited predicted amino acid sequences not starting with a methionine (Online Resource 1). Therefore, it is likely that these sequences are incomplete at the 5' end, not permitting identification of a signal peptide in the truncated sequence. This was also the case for some Norway spruce sequences.

A second tree comprising only white spruce chitinase sequences allowed us to map predicted protein domains in the context of phylogenetic relationships within this gene family (Fig. 2). The topology of this tree was consistent with the larger tree, showing five distinct groups of white spruce genes within the five major clusters, with two subclusters evident within cluster 1.

White spruce and Norway spruce chitinases were assigned to classes using both the position within the tree and the presence of diagnostic domains according to Neuhaus (1999). The classification scheme is outlined in Online Resource 3. Cluster 1 contained several well-characterized class IV chitinases from other species. All full-length white spruce and Norway spruce sequences from cluster 1 that exhibited a chitin binding

Fig. 1 Neighbor-joining tree showing the relationships of chitinases from white spruce, Norway spruce, and selected other plant species. The first two letters in each sequence identifier represent the species, followed by the GenBank gene ID (gi) for sequences with known functions, the full-length insert cDNA (FLIC) ID for the white spruce sequences, or the v1.0 gene model ID for the Norway spruce sequences. *At*, *Arabidopsis thaliana*; *Zm*, *Zea mays*; *St*, *Solanum tuberosum*; *Ca*, *Capsicum annuum*; *Os*, *Oryza sativa*; *Rg*, *Rehmannia glutinosa*; *Gh*, *Gossypium hirsutum*; *Ta*, *Triticum aestivum*; *Vv*, *Vitis vinifera*; *Nt*,

Nicotiana tabacum; *La*, *Lupinus albus*; *Hv*, *Hordeum vulgare*; *Pg*, *Picea glauca*; *Cd*, *Cynodon dactylon*; *Sc*, *Secale cereale*; *Ma*, *Musa acuminata*; *Sr*, *Sesbania rostrata*; *Ms*, *Medicago sativa*; *Pa*, *Picea abies*; *Pm*, *Pseudotsuga menziesii*; *Vc*, *Vaccinium corymbosum*. For the previously characterized chitinases from other plant species, the reported chitinase structural class is indicated at the end of the sequence identifier in parentheses. Within this tree, the chitinases fall into two main groups according to their major catalytic domain, the glycoside hydrolase family 18 (GH 18) and glycoside hydrolase family 19 (GH 19)

domain (CBD, Fig. 2, Online Resource 3) are likely to be class IV chitinases, based on the presence of the CBD, linker sequence, and greatest sequence similarity in the catalytic domain to class IV chitinases of other species (Neuhaus 1999). These grouped together into one subcluster of cluster 1 (Fig. 1). Several white spruce and Norway spruce sequences falling into the other subcluster of cluster 1 showed high sequence similarity within the catalytic domain to class IV chitinases but lacked a CBD. In older chitinase nomenclature

schemes, these would have been designated class II chitinases, on the basis of the absent CBD (Hamel et al. 1997). However, according to the newer naming convention put forth by the Chitinase Nomenclature Commission, chitinases showing homology to class IV catalytic domains but lacking a CBD should be classified as class VII (Neuhaus 1999). Accordingly, these white spruce and Norway spruce sequences which would be otherwise classified as class II by similarity to other plant chitinases previously classified as

Fig. 2 Predicted structural domains of white spruce chitinases, grouped according to a phylogenetic tree constructed from the full deduced amino acid sequence of each white spruce chitinase. Bootstrap values for the neighbor-joining tree are given for the major branches on the phylogenetic tree. The Arabic numbers to the left of the tree correspond to the cluster designations from Fig. 1. The predicted structural domains

for each of the white spruce sequences in the phylogenetic analysis are also indicated. The different structural domains corresponding to each sequence are depicted in the figure legend: *arrow* signal peptide domain, *thick bar* CBD, *thin bar* linker domain, *rounded rectangle* catalytic domain

class II (see Fig. 1) are likely better designated as class VII chitinases.

Similar to cluster 1, cluster 2 contained white spruce chitinases with and without a CBD. This cluster also included a number of well-characterized class I and class II chitinases from other species. A CBD was identified for three white spruce sequences within one of the

subclusters of cluster 2. Given the sequence similarity between the known class I chitinases and these chitinases, together with the presence of a CBD, these three chitinases are likely to be class I chitinases. Those chitinases that showed similarity to class I chitinases but lacked the CBD fall into a different sub-cluster and are more likely to be class II chitinases

Fig. 3 Topology of relationships among predicted protein tertiary structures for white spruce chitinases. Structures were compared using the FAST algorithm from the ProCKSI-Server (<http://www.procksi.net/>).

The distance matrix of RMSDs of all pairwise comparisons was standardized and used to cluster sequences and create the tree

(Fig. 1, Online Resource 3). No cluster 2 Norway spruce sequences exhibited a CBD, and none were found in this subcluster.

3D protein models were obtained by homology modeling for 30 white spruce predicted proteins. The predicted tertiary structures were aligned to each other using the FAST algorithm (Zhu and Weng 2005) to obtain an RMSD matrix of all pairwise comparisons (Online Resource 4), which were used to cluster the proteins according to their similarities (Fig. 3). The chitinases belonging to the GH 18 family showed a distinct (α/β) eight barrel fold, in contrast to the GH 19 family members, which exhibited structures rich in α -helices and included a distinct catalytic cleft. While the cluster 4 (class III) and cluster 5 (class V) chitinase sequences belonging to GH 18 were clustered similarly according to these predicted tertiary structures as they did for the phylogenetic analysis based on conventional primary sequence alignments (cf. Figs. 2 and 3), GH 19 chitinases exhibited somewhat different groupings in the former than the latter that reflected the presence of common domains, particularly the CBD. A distinctive characteristic of the structural clustering was that putative class I and class IV structures bearing the CBD clustered together, showing that this domain allowed for additional structural matching when performing the alignments (Fig. 3). Most putative class VII chitinases from cluster 1 grouped together in this structure-based tree, while the putative class II chitinases from cluster 2 did not form a distinct group.

Interestingly, the CBD region of each class I and class IV chitinase was predicted to have a high degree of intrinsic disorder (Online Resource 5). A comparable IDR was not detected in sequences lacking this domain. A small number of sequences from clusters 2 and 4 exhibited regions within the C-terminal half of the catalytic region showing lesser degrees of intrinsic disorder. Additionally, all sequences exhibited a short IDR at the C-terminal, while all full-length sequences exhibited a short IDR at the N-terminal that corresponded to the predicted signal peptide domain.

Characterization of autumnal acclimation-associated white spruce chitinases

Classification

The eight chitinases previously identified as differentially expressed in white spruce stems during the activity-dormancy transition (Galindo-González et al. 2012) fall into cluster 1 (Pg_GQ03206_D15.1, Pg_GQ03803_J20.1, Pg_GQ0207_E09.1), cluster 2 (PgGQ0183_A06.2, Pg_GQ03904_P01.1, Pg_GQ03703_O09.1, Pg_GQ03902_B01.1), and cluster 3 (Pg_GQ0046_J03.1) of

Fig. 1. No genes identified as differentially expressed by Galindo-González et al. (2012) belonged to the GH 18 clusters 4 or 5. The clustering of these genes with previously characterized chitinases from other species (Online Resource 6) and the presence or absence of a CBD suggests that Pg_GQ03206_D15.1 and Pg_GQ03803_J20.1 (cluster 1) are class IV chitinases; Pg_GQ0207_E09.1 (cluster 1) is a class VII chitinase; PgGQ0183_A06.2 and Pg_GQ03904_P01.1 (cluster 2) are class I chitinases; Pg_GQ03703_O09.1 and Pg_GQ03902_B01.1 (cluster 2) are class II chitinases; and Pg_GQ0046_J03.1 (cluster 3) is a class II chitinase or chitinase-like (CTL) sequence (sensu Zhang et al. 2004; Sánchez-Rodríguez et al. 2012).

To further resolve the classification of these dormancy-associated chitinases, the sequences were examined in more detail by alignment to a class I tobacco chitinase (Neuhaus et al. 1991) that exhibits all of the canonical chitinase motifs in order to identify motifs important to their classification (Fig. 4). We used the structural classification characters outlined by Hamel et al. (1997) and Neuhaus (1999) to guide the classification. The alignment showed that Pg_GQ0183_A06.2 and Pg_GQ03904_P01.1—which showed similar domain features—shared more sequence similarity to the tobacco chitinase than the others (62 and 66 %, respectively, identity while the remaining six sequences ranged from 33 to 58 %; Online Resource 7), providing additional evidence that these two sequences are class I chitinases. In particular, the full-length Pg_GQ0183_A06.2 exhibits the N-terminal CBD separated from the catalytic domain by a linker sequence, characteristic of class I chitinases (Hamel et al. 1997; Neuhaus 1999). Pg_GQ03904_P01.1, missing the 5' region of the sequence, does not exhibit these motifs (probably due to an incomplete sequence) but shows high sequence similarity within the catalytic domain. Pg_GQ03703_O09.1 and Pg_GQ03902_B01.1 (Fig. 4) likely belong to class II because they each lack a CBD and carboxy-terminal extension (CTE), and they exhibit a deletion in loop 2 of the catalytic domain of 17 and 14 amino acids, respectively (Hamel et al. 1997). Chitinases Pg_GQ3206_D15.1 and Pg_GQ3803_J20.1 did not exhibit any deletions in loop 2. Instead, these sequences exhibit a 13 amino acid deletion in loop 1 of the catalytic domain and 5 and 13 amino acid

Fig. 4 Motif identification in dormancy-associated white spruce chitinases. The white spruce chitinase sequences were aligned to a class I chitinase from tobacco, since this sequence has all features of a traditional class I chitinase, plus a carboxy terminal extension which has been linked to vacuolar localization (Neuhaus et al. 1991). Identical residues in all sequences are highlighted in a darker shade, while conserved and similar residues are demarcated in the corresponding lighter shade. Shaded blocks represent domains: signal peptide (green), CBD (blue), catalytic domain (red), and carboxy terminal extension (yellow). Significant deletions are boxed

		1		60
Nt_gi_19861 (I)	(1)	-----MRLCKFTALSSLLFSLLLLSASAEQCSQAAGGARCPSSLCCSKFGWCGNTN		
Pg_GQ03904_P01.1 (I)	(1)	-----MRLCKFTALSSLLFSLLLLSASAEQCSQAAGGARCPSSLCCSKFGWCGNTN		
Pg_GQ0183_A06.2 (I)	(1)	LTSVREMKSIRFSAMAIALVTGMTNLYFASAEQCGRQASGALCPGGLCCSKWGWCGNTE		
Pg_GQ03902_B01.1 (II)	(1)	-----RGTRICIGSLKMASATIGRMKSMRVLVLAAMMGTLCHVSAQQG---		
Pg_GQ03703_O09.1 (II)	(1)	-----MRVLLALTIVVAMMN-TFCYVSAQQG---		
Pg_GQ0046_J03.1 (II)	(1)	-----METKQIIGSVNRMVLVVVGICLMISSWLCCIEPAAAADED		
Pg_GQ0207_E09.1 (VII)	(1)	-----TILANMAIHLRVMTASVFVLWALAL-----SICRG---		
Pg_GQ03803_J20.1 (IV)	(1)	---MAGSSGKFASPRGRVVVRMSLVLLLVGVSVNVVNAQNCGCASGLCCSKWGYCGTTS		
Pg_GQ03206_D15.1 (IV)	(1)	---MGSSSSNKS-----VMAVLVLVLLLVGVSVN---AQNCGCASGVCCSQYGYCGTTS		
		61		120
Nt_gi_19861 (I)	(52)	DYCGPGNCQSQCPGGPTPTPPTPPGGGLGSIISSSMFDQMLKHRNDNACQKGFYSYNA		
Pg_GQ03904_P01.1 (I)	(1)	-----FIEAQRRRRCKAKGFYSYAA		
Pg_GQ0183_A06.2 (I)	(61)	AHCG-QDCQSQCGGSTPTPPSPTPGGQGVSSVITESIFNDLLKHRNDACKAKGFYSYAA		
Pg_GQ03902_B01.1 (II)	(48)	-----VASIISEDVFNQFLKHRNDACPAKGFYTYS		
Pg_GQ03703_O09.1 (II)	(25)	-----VAFIITEDVFNRFLKHRNDGACAAGKGFYTYS		
Pg_GQ0046_J03.1 (II)	(40)	QAMK-----SKKIACIKGAECKNKTISELFTVDQFESLFSHRNAPMAHAQGFWDYHS		
Pg_GQ0207_E09.1 (VII)	(32)	-----DVGDIAEDFFNGILSG-AADTCAGKKFYTYS		
Pg_GQ03803_J20.1 (IV)	(58)	AYCGNGCQSQGPCS---GGGSPSGGGGNVGTIIISQNFNSLASG-AGSSCKGKGFYTYS		
Pg_GQ03206_D15.1 (IV)	(48)	AYCGKGCKSGPCYSS---GGGSPSAGGGSVGGIIISQSFNGLAGG-AGSSCEGKGFYTYS		
		121	Loop 1	180
Nt_gi_19861 (I)	(112)	FINAARSFP--GFGTSGDTTARKREIAAFFAQTSHETTGGWATAP-----DGPYAWGYC		
Pg_GQ03904_P01.1 (I)	(21)	FIAAADAFP--GFGTTGDLTAQKRELAFFGQTSHETTGGWPTAP-----DGPYAWGYC		
Pg_GQ0183_A06.2 (I)	(120)	FIAAANAFP--AFGTTGDLSTRKRELAFFGQTSHETTGGWPAAP-----DGPYAWGYC		
Pg_GQ03902_B01.1 (II)	(80)	FIAAANSFP--DFGNNGDLETSKRELAFFGQTSQETTGGWATAP-----DGPYAWGYC		
Pg_GQ03703_O09.1 (II)	(57)	FILAANRFP--SPGNVGNLETRKRELAFFAQTSHETTGGWETAP-----DGPYAWGYC		
Pg_GQ0046_J03.1 (II)	(92)	FITAAAHFEPKGFATGDLVQKELAAFFAHVATETSCESLMAQSSTATDTPSKWGLC		
Pg_GQ0207_E09.1 (VII)	(64)	FITAANGFS--GFGTTGSDDEKREIAAFFANVAHETIG-----FC		
Pg_GQ03803_J20.1 (IV)	(113)	FIAAANAYS--GFGTTGSNEVQKRELAFFANVMHETGG-----LC		
Pg_GQ03206_D15.1 (IV)	(105)	FIAAANAFS--GFGTTGSNDVKKRELAFFANVMHETGG-----LC		
		181	Loop 2	240
Nt_gi_19861 (I)	(164)	WLREQGSF--GDYCTPSGQWPCAPGRKYFGRGPIQISHNYNYGPCRAIGVDLLNPNDLVA		
Pg_GQ03904_P01.1 (I)	(73)	LKEEQGDPPEGEYQASSEWPCASGKKYYGRGPVQISWNYNYGPAGKAVGFDGINNPDIIA		
Pg_GQ0183_A06.2 (I)	(172)	FKEEQGNPPGEYQASSQWPCASGKRYGRGPVQISWNYNYGPAGRAIGFDGINNPDIVA		
Pg_GQ03902_B01.1 (II)	(132)	FKEE-----NSADRYHGRGPIQLTGDYNYKAAGDALGYDLINNPGLVA		
Pg_GQ03703_O09.1 (II)	(109)	FKEEOD-----ADPEEMYHGRGPIQLTGKSNYEAAKGALGYDLINNPGLVA		
Pg_GQ0046_J03.1 (II)	(152)	YKEELS--PDSTYCESSLVPCAPGVSYHGRGALPVYWNVNYGQQLQALKVDLLHHAEDLS		
Pg_GQ0207_E09.1 (VII)	(103)	YIEEIQ--KDDYCDNNQYPCAAAGQVYGRGPIQLSWNFNYGPAGSYLQVLDLLNPNPETVA		
Pg_GQ03803_J20.1 (IV)	(152)	YINEIS--PSSNYQSSSTWPCSTGKSYHGRGPIQLSWNFNYGAAGQSIGFDGLNNPEKVG		
Pg_GQ03206_D15.1 (IV)	(144)	YINEIN--PKIYQSSSTWPCSTGKSYHGRGPIQLSWNFNYGAAGKSIGFDGLNNPEKVG		
		241	Loop 3	300
Nt_gi_19861 (I)	(223)	TDPVISFKSALWFMTQSP--KPSCHDVIIGRWQPSAGDRAANRLPGFGVITNIINGGLE		
Pg_GQ03904_P01.1 (I)	(133)	NDATVSFKTAVWFMTQSP--KPSCHNVMAAGWGPGSGSDTAAGRAAGYGVVTNIINGGLE		
Pg_GQ0183_A06.2 (I)	(232)	NDATVSFKTAMWFMTQSP--KPSCHDVTGRWSPSGRDSAPGRAAGYGVVTNIFNGGLE		
Pg_GQ03902_B01.1 (II)	(175)	TDATVSFKTALWFMTQSP--KPSCHDVLGRWSPSDTDAAGRVPGYGMVTNIINGGLE		
Pg_GQ03703_O09.1 (II)	(155)	SNPTISFKTAVWFMTQSP--KPSPTVMIGKWSPGSGSDTAAGRVPGYGVVTNIINGGVE		
Pg_GQ0046_J03.1 (II)	(211)	QNATLAFQAAMWRWMNPIKVKQPSAHQVMVGKWPVKNDTNSLRHPFGMTINILKGDAL		
Pg_GQ0207_E09.1 (VII)	(162)	QDALIAWKTALWYNNV-----NSDCHSAITSGQ-----GFGATIQAINGALE		
Pg_GQ03803_J20.1 (IV)	(211)	QDATISFKTAVWFWMK-----NSNCHSAITSGQ-----GFGGTIKAIN-SGE		
Pg_GQ03206_D15.1 (IV)	(203)	QDSTISFKTAVWFWMK-----NSNCHSAITSGQ-----GFGGTIKAIN-NME		
		301		358
Nt_gi_19861 (I)	(282)	CGRGTDSRVQ--DRIGFYRRYCSILGVSP---GDNLDGNGQSRFNG-----LLVDTM-		
Pg_GQ03904_P01.1 (I)	(192)	CGKGSDSRQE--DRIGFYKRYCDILGVSY---GSNLDGNSQKPFGFAAQSPRLIKTVV		
Pg_GQ0183_A06.2 (I)	(291)	CGKGSDSRQV--DRIGFYKRYCDIFGVRY---GSNLECNQKPFGFAAQSPPHLTKTVV		
Pg_GQ03902_B01.1 (II)	(234)	CGQGTSSAAQQRIGFYQTYCNKLGVD---GSNLDGNNQKPFGS-----		
Pg_GQ03703_O09.1 (II)	(214)	CGRGSDSNQE--DRIGFYKAYCDILGASY---GSNVDCNTQSRFSG-----		
Pg_GQ0046_J03.1 (II)	(271)	CGAGSDDKQMNKRIAHLYFLDQLDVGRDNAGDNLDCSDQKVLNPSSAST-----		
Pg_GQ0207_E09.1 (VII)	(204)	CNGGNTDEVN--DRVSYTYKCSQFGVDP---GSNLSC-----		
Pg_GQ03803_J20.1 (IV)	(252)	CNGGNSGQVN--SRVTYYKKFCSQLGVDT---GTNVSC-----		
Pg_GQ03206_D15.1 (IV)	(244)	CNGGNSGEVS--SRVNYKKIKCSQLGVDP---GVNVSC-----		

deletions within and flanking loop 3 of the catalytic domain. Loss of loop 1 is characteristic of class IV chitinases (Neuhaus

1999). Additionally, Pg_GQ3206_D15.1 and Pg_GQ3803_J20.1 had no identifiable CTE, but each

exhibited a CBD, which is also characteristic of class IV chitinases. Pg_GQ0207_E09.1 did not show evidence of a CBD or CTE but is more similar to class IV than class I chitinases. This is consistent with its classification as a class VII chitinase based on the scheme outlined by Neuhaus (1999). Furthermore, Pg_GQ0207_E09.1 exhibited the identical deletions associated with loop 2 and loop 3 as the class IV chitinases Pg_GQ3206_D15.1 and Pg_GQ3803_J20.1. Pg_GQ0046_J03.1 did not exhibit deletions within the catalytic domain, although there was a six amino acid insertion in loop 1. Pg_GQ0046_J03.1 further displayed no evidence of a CBD or CTE, and showed a low sequence identity when compared to the other white spruce sequences (Online Resource 7). This sequence showed the most similarity to the *A. thaliana* sequences encoding CHITINASE-LIKE 1 (CTL1) and CHITINASE-LIKE 2 (CTL2), which are designated class II chitinases, although they lack chitinase activity (Zhang et al. 2004; Hermans et al. 2010; Sánchez-Rodríguez et al. 2012). Both AtCTL1 and AtCTL2 lack the highly conserved histidine-glutamic acid-threonine-threonine (HETT) motif within loop 1 that is essential for chitinase activity (Passarinho and de Vries 2002). Pg_GQ0046_J03.1 also lacks this HETT motif, which is replaced by threonine-glutamic acid-threonine-serine (TETS). Thus, Pg_GQ0046_J03.1 could be considered a class II chitinase or CTL.

These more detailed sequence analyses provide further support for the class designations made in Figs. 1 and 2 and Online Resource 3, including the distinct clustering of class IV and class VII (presently called class II) genes within cluster 1, and distinct clustering of class I and class II genes within cluster 2.

qRT-PCR expression profiling

Quantitative RT-PCR was used to generate transcript profiles for each of these eight sequences in shoot tips, needles, stems, and roots during the short day-induced transition from active growth to dormancy (Fig. 5). Based upon the analyses conducted in El Kayal et al. (2011) and Galindo-González et al. (2012), bud formation was nearly complete after 70-day short day treatment and was completed before the trees were transferred to 4 °C. Neither short days nor cold temperatures are necessary to achieve cold tolerance or transition to dormancy in these seedlings, although short days accelerate these processes (Hamilton, El Kayal, and Cooke, unpublished). Six of the eight sequences showed statistically significant changes in transcript abundance in stems, the tissue in which they were originally identified as differentially expressed by microarray or proteomics analysis (Galindo-González et al. 2012). Each of these six sequences also showed statistically significant differences in transcript abundance in at least one other tissue. Most of these genes showed upregulation during the latter stages of autumnal acclimation. A notable exception was

Pg_GQ0046_J03.1, which showed significant reductions in transcript abundance in all tissues over time. While some closely related genes exhibited very similar transcript profiles, e.g., the cluster 2/class I Pg_GQ0183_A06.2 and Pg_GQ03904_P01.1, other closely related genes, did not exhibit similar patterns of transcript abundance. For example, while the cluster 1/class IV chitinases Pg_GQ3206_D15.1 and Pg_GQ3803_J20.1 were closely related (Figs. 1 and 2), Pg_GQ3206_D15.1 was much more highly expressed than Pg_GQ3803_J20.1, and only the former showed significant changes over time in shoot tips, needles, and stems (Fig. 5). Similarly, the cluster 2/class II Pg_GQ03703_O09.1 showed strong upregulation in all tissues over time, while the closely related cluster 2/class II Pg_GQ03902_B01.1 showed very low transcript abundance with no significant differences in any tissue over time.

In silico analyses

We performed an in silico prediction of putative active site residues using COFACTOR. For this analysis, we used the five top closest enzyme homologs determined by structural alignment to determine the most likely residues involved in the active site (Online Resource 8). If the residue was part of the active site in four of the five alignments for each of the spruce chitinases, it was classified as a predicted active site residue (Fig. 6). When the modeled sections of the proteins were aligned, it was shown that the predicted active site amino acids were conserved (Holm and Sander 1994; Hart et al. 1995). For each of the proteins, two glutamic acids were predicted to constitute part of the active site (Fig. 6), but a serine that was predicted to be part of the active site was only present in five of the eight sequences: Pg_GQ03904_P01.1, Pg_GQ0183_A06.2, Pg_GQ0207_E09.1, Pg_GQ03803_J20.1, and Pg_GQ03206_D15.1. Three amino acids (Gln, Tyr, and Asn) important for substrate binding (Verburg et al. 1993; Hart et al. 1995) were conserved in five sequences, while Pg_GQ03703_O09.1 exhibited a Ser in place of the Tyr, Pg_GQ0207_E09.1 exhibited a Phe in place of the Tyr, and Pg_GQ0046_J03.1 exhibited a Pro in place of the Gln. In summary, four sequences exhibited all six active site residues: Pg_GQ03206_D15.1 (cluster 1/class IV), Pg_GQ03803_J20.1 (cluster 1/class IV), Pg_GQ0183_A06.2 (cluster 2/class I), and Pg_GQ03904_P01.1 (cluster 2/class I). However, only the two cluster 2/class I sequences exhibit the HETT motif found to be essential for activity (Passarinho and de Vries 2002), whereas the two cluster 1/class IV sequences exhibit a substitution in this motif.

We also carried out in silico analyses of protein properties consistent with an antifreeze function. Prediction of putative ice binding residues using AFPredictor (Doxey et al. 2006) was performed for the eight dormancy-associated white spruce chitinases and four plant chitinases from other species known to be involved in cold acclimation (Table 1). The

Fig. 5 Quantitative RT-PCR transcript abundance of eight white spruce chitinases previously demonstrated to be differentially expressed in stems during the SD-induced activity-dormancy transition (Galindo-González et al. 2012). The cluster and putative biochemical class is indicated above the sequence identifier. Expression was determined relative to *TIF5A*; both the target and reference genes were quantified using standard curves. Values represent the mean $\pm$ SE, $n=4$. For data analyzed by GLM, data were first log transformed to meet the

assumptions of normality and homogeneity of variance. For each gene, different letters denote statistical difference within a tissue at $P<0.05$. In the cases of stem tissue data for Pg_GQ0183_A06.2 and Pg_GQ03703_O09.1, transformation was insufficient to meet assumptions of normality and homogeneity of variance required for parametric tests. Here, data were analyzed using a non-parametric test as described in “Materials and methods,” and significant differences within a tissue at $P<0.05$ are indicated with asterisks

analysis predicted that two of the four chitinases involved in cold acclimation (one from *Vaccinium corymbosum* and one from *Secale cereale*) contain OSCs, indicative of ice-binding surfaces (Doxey et al. 2006). Likewise, six out of the eight

white spruce chitinases also presented OSCs. The total surface area (TSA) of ice binding was higher in five of the six white spruce proteins relative to the previously characterized proteins from blueberry and rye, and two white spruce proteins

		1	60
Pg_GQ03904_P01.1 (3D)	(1)	-----	-----
Pg_GQ0183_A06.2 (3D)	(1)	EQCGRQASGALCPGGLCCSKWGCNTEAHCGQDCQSQCGGSTPTPPSPTPGGQCVSSVI	
Pg_GQ03902_B01.1 (3D)	(1)	-----	-----GVASII
Pg_GQ03703_O09.1 (3D)	(1)	-----	-----VAFII
Pg_GQ0046_J03.1 (3D)	(1)	-----	-----KTISELF
Pg_GQ0207_E09.1 (3D)	(1)	-----	-----VGDI A
Pg_GQ03803_J20.1 (3D)	(1)	-----AQNCGCASGLCCSKWGYCGTTSAYCGNGCQSGPCS---	GGGSPSGGGNVGTII
Pg_GQ03206_D15.1 (3D)	(1)	-----AQNCGCASGVCCSQYGYCGTTSAYCGKCKSGPCYSS-	GGGSPSAGGGSVGGII
		61	120
Pg_GQ03904_P01.1 (3D)	(1)	-----CKAKGFYSYAAFI A A A D A F P - -	GFGTTGDLTAQKRELA A A F G Q T
Pg_GQ0183_A06.2 (3D)	(61)	TESIFNDLLKHRNDAACKAKGFYSYAAFI A A A N A F P - -	AFGTTGDLSTRKRELA A A F G Q T
Pg_GQ03902_B01.1 (3D)	(7)	SEDVFNQFLKHRNDACPAKGFYTSAFI A A A N S F P - -	DGNNGLDLETSKRELA A A F G Q T
Pg_GQ03703_O09.1 (3D)	(6)	TEDVFNRLKHRNDGACAAKGFYTSNFI A A A N R F P - -	SFGNVNLETRKRELA A A F A Q T
Pg_GQ0046_J03.1 (3D)	(8)	TVDOFESLFSHRNAPMAHAQGFWDVHSFI T A A A H F E P K G F G A T G	GDLVQKKRELA A A F F A H V
Pg_GQ0207_E09.1 (3D)	(6)	TEDFENGILSG-AADTCAGKKFYTSDFI T A A A N G F S - -	GFGTTGTSDEKRELA A A F F A N V
Pg_GQ03803_J20.1 (3D)	(52)	SQNFNLSASG-AGSSCKGKGFYTSNFI A A A N A Y S - -	GFGTTGSNEVQKRELA A A F F A N V
Pg_GQ03206_D15.1 (3D)	(54)	SQSFFNGLAGG-AGSSCEGKGFYTSNFI A A A N A F S - -	GFGTTGSNDVKKRELA A A F F A N V
		121	180
Pg_GQ03904_P01.1 (3D)	(43)	SHETGGWPTAF-----	DGPYAWGYCLKEEQGDPPGEYCOASSEWPCASGKKYYGRGPV
Pg_GQ0183_A06.2 (3D)	(119)	SHETGGWPAAP-----	DGPYAWGYCFKEEQGNPPGEYCOASSQWPCASGKYYGRGPV
Pg_GQ03902_B01.1 (3D)	(65)	SOETGGWATAF-----	DGPYAWGYCFKEE-----NSADRHHGRGPI
Pg_GQ03703_O09.1 (3D)	(64)	SHETGGWETAF-----	DGPYAWGYCFKEEOD-----ADPEEMYYGRGPI
Pg_GQ0046_J03.1 (3D)	(68)	ATETSCESLMAQSSTATTDSPTKWGLCYKEELS-	PDSTYCESSLVYPCAPGVSVHGRGAL
Pg_GQ0207_E09.1 (3D)	(63)	AHETIG-----	FCYIEI IQ-KDDYCDNNQYPCAAQKQYYGRGPI
Pg_GQ03803_J20.1 (3D)	(109)	MHETGG-----	LCYINELIS-ESSNYCQSSSTWPCSTSGKSVHGRGPL
Pg_GQ03206_D15.1 (3D)	(111)	MHETGG-----	LCYINELIN-EKIIYCQSSSTWPCSTSGKSVHGRGPL
		181	240
Pg_GQ03904_P01.1 (3D)	(97)	QISWNNYGPAGKAVGF	GINNPDIANDATVSFKTAVVFWMTQSPK-PSCHNVMAAGW
Pg_GQ0183_A06.2 (3D)	(173)	QISWNNYGPAGRAIGF	GINNPDIIVANDATVSFKTAMVFWMTAQSPK-PSCHVMTGRW
Pg_GQ03902_B01.1 (3D)	(102)	QLTGDNVKAAGDALGYDLINNPVLVVT	DATVSFKTALVFWMTQSPK-PSCHVILGRW
Pg_GQ03703_O09.1 (3D)	(104)	QLTGKSNVEAAGKALGYDLLNPGIVASNP	TISFKTAVVFWMTQSPK-PSCHVTVMIGKW
Pg_GQ0046_J03.1 (3D)	(127)	PVYWNVNYGQLQALKVLLHHAEDLSQ	NATLAFQAAMRWNNPIKVQPSAHQVMVQKW
Pg_GQ0207_E09.1 (3D)	(103)	QLSWNFNYGPAGSYLGVDLLNPE	TVAQDALIAWKALVYNNV-----NSDCHSAITSGQ
Pg_GQ03803_J20.1 (3D)	(149)	QISWNNYGAAGQSIGF	GLNNPEKVGQDATISFKTAVVFWMK-----NSNCHSAITSGQ
Pg_GQ03206_D15.1 (3D)	(151)	QLSWNNYGAAGKSIGF	GLNNPEKVGQDSTISFKTAVVFWMK-----NSNCHSAITSGQ
		241	300
Pg_GQ03904_P01.1 (3D)	(156)	GPSGSDTAAGRAAGYGVVTNI	INGGLECGKGSDSRQE-DRIGFYKRYCDILGVSY---GS
Pg_GQ0183_A06.2 (3D)	(232)	SPSGRDSAPGRAAGYGVVTNI	IFNGGLECGKGSDSRQV-DRIGFYKRYCDIFGVRY---GS
Pg_GQ03902_B01.1 (3D)	(161)	SPSDTDTAAGRVP	GYGMVTNIINGGLECGQGTSSAAQQGRIGFYQTYCNKLGVDSD---GS
Pg_GQ03703_O09.1 (3D)	(163)	SPSGSDTAAGRVP	GYGVVTNIINGGVECGRGSDSNQE-DRIGFYKAYCDILGASY---GS
Pg_GQ0046_J03.1 (3D)	(187)	VPTKNDTNSLRHP	FGGMTINILKGDAECGAGSDDKQMNKRIAHYLYFLDQLDVGRDNAGD
Pg_GQ0207_E09.1 (3D)	(156)	-----	GFGATIQAINGALECNGCNTDEVN-DRVSYTKYCSQFGVDP---GS
Pg_GQ03803_J20.1 (3D)	(202)	-----	GFGGTIKAIN-SGECNCGNSGQVN-SRVYTKKFCSQLGVDT---GT
Pg_GQ03206_D15.1 (3D)	(204)	-----	GFGGTIKAIN-NMECNGNSGEVS-SRVNYKKICSQLGVDP---GV
		301	311
Pg_GQ03904_P01.1 (3D)	(212)	NLDNSQKPF	FG
Pg_GQ0183_A06.2 (3D)	(288)	NLECNTQKPF-	
Pg_GQ03902_B01.1 (3D)	(218)	NLDNNQKPF	FG
Pg_GQ03703_O09.1 (3D)	(219)	NVDNNTQRS	FG
Pg_GQ0046_J03.1 (3D)	(247)	NLDSDQKVLN	
Pg_GQ0207_E09.1 (3D)	(201)	NLSC-----	
Pg_GQ03803_J20.1 (3D)	(246)	NVSC-----	
Pg_GQ03206_D15.1 (3D)	(248)	NVSC-----	

Fig. 6 Depiction of predicted active site amino acids in dormancy-associated white spruce chitinases. *Black circles* below sequences show predicted active site amino acids by structural alignment of white spruce sequences based on predicted 3D models to the top five closest enzyme homologs using COFACTOR (<http://zhanglab.cmb.med.umich.edu/COFACTOR/>). An *open circle* is used to indicate the predicted glutamic acid that was only present in two of the five top enzyme homologs (see Online Resource 8). *Plus signs* indicate amino acids important in substrate binding (Hart et al. 1995)

had TSA values over 200 Å. The fraction of surface area (FSA) was likewise higher in the white spruce proteins. The two white spruce proteins with the higher TSA—Pg_GQ03803_J20.1 and Pg_GQ03902_B01.1—also exhibited the higher FSAs (0.021 and 0.025). When the white spruce sequences from the predicted 3D models bearing OSCs were aligned (Online Resource 9), it was evident that many of the amino acids which made up the predicted ice-binding surfaces were conserved between sequences. For Pg_GQ03206_D15.1 and Pg_GQ03803_J20.1, the amino acids Cys, Gly, Ala, and Ser were conserved, while for Pg_GQ03703_O09.1, Pg_GQ03902_B01.1, and Pg_GQ03904_P01.1, the amino acids Thr, Ala, Gly, and Val/Ala were also conserved among the three sequences.

Digital expression profiling of spruce chitinases

We used transcriptome data derived from ca. 6.6 million Roche 454 reads representing seven different white spruce tissue libraries—vegetative buds, current year foliage, mature tree xylem, juvenile tree xylem, juvenile tree bark, megagametophytes, and somatic embryogenic tissue—to further characterize gene expression for the eight dormancy-associated white spruce chitinases (Online Resource 10). These libraries are described in detail in Raherison et al. (2012). Five of the eight genes—GQ03803_J20, GQ0183_A06, GQ03904_P01, GQ_03902_B01, and GQ03703_O09—were expressed in nearly all tissues. These five genes and GQ0046_J03 were all found to be expressed in vegetative buds, consistent with our data. GQ03206_D15 was not found to be expressed in vegetative buds in this transcriptomic dataset. Several other chitinases representing all classes and clusters were also found to be expressed in vegetative buds.

Transcripts for GQ0207_E09 were only noted in the somatic embryogenic tissue. Two thirds of the chitinases were expressed in embryogenic tissue, more than in any other tissue that was surveyed. Chitinases tended to be more highly expressed in embryogenic tissue than other tissues.

We also carried out digital expression profiling in ConGenIE of RNASeq data for 22 libraries (Nystedt et al. 2013). Fifty-six of the 76 Norway spruce sequences were represented in this dataset. At least one chitinase showed high transcript abundance for 18 of the 22 libraries (Online Resource 11). Clustering revealed co-regulated groups of

Table 1 Prediction of ice-binding surfaces for four previously characterized chitinases associated with cold acclimation and eight dormancy-associated white spruce chitinases

Protein ID	Species	No. OSCs	OSC aa	TSA	FSA
Cd_gi 4008072	Cynodon dactylon	0	N/A	0.000	0.000
Sc_gi 12407649	Secale cereale	0	N/A	0.000	0.000
Sc_gi 12407647	Secale cereale	4	Ser48 (CB), Cys51 (CB), Gly52 (CA), Cys59 (CB)	105.167	0.008
Vc_gi 224775714	Vaccinium corymbosum	4	Gly68 (CA), Thr72 (CG2), Val71 (CG2), Pro249 (CB)	101.536	0.010
Pg_GQ0046_J03.1	Picea glauca	0	N/A	0.000	0.000
Pg_GQ0183_A06.2	Picea glauca	0	N/A	0.000	0.000
Pg_GQ03904_P01.1	Picea glauca	5	Thr171 (CB), Thr171 (CG2), Ala172 (CB), Gly174 (CA), Ala176 (CB)	160.844	0.015
Pg_GQ03902_B01.1	Picea glauca	7	Ser209 (CB), Thr211 (CG2), Thr213 (CB), Thr213 (CG2), Ala214 (CB), Gly216 (CA), Val218 (CG2)	259.908	0.025
Pg_GQ03703_O09.1	Picea glauca	4	Thr193 (CB), Ala194 (CB), Gly196 (CA), Val198 (CG2)	104.557	0.010
Pg_GQ0207_E09.1	Picea glauca	4	Thr66 (CG2), Asn69 (CB), Gly70 (CA), Ser72 (CB)	122.392	0.013
Pg_GQ03803_J20.1	Picea glauca	8	Cys39 (CB), Gly40 (CA), Ala42 (CB), Ser43 (CA), Gly44 (CA), Gly67 (CA), Cys69 (CB), Ser70 (CB)	262.202	0.021
Pg_GQ03206_D15.1	Picea glauca	4	Cys29 (CB), Gly30 (CA), Ala32 (CB), Ser33 (CA)	126.391	0.010

The TSA is the surface area in angstroms from the protein 3D model which is covered by the predicted ice-binding surface; the FSA is the portion of surface area in the protein 3D model which is covered by the predicted ice-binding surface. Acronyms in parenthesis after the amino acids carrying the OSCs correspond to the backbone carbons: carbon alpha, carbon beta, and carbon gamma. OSCs: ordered surface carbons, aa amino acids, TSA total surface area, CA carbon alpha, CB carbon beta, CG carbon gamma

chitinases, although there was no clear relationship between co-regulation and biochemical class or phylogenetic cluster. Every sequence exhibited high transcript abundance under at least one condition, but few sequences exhibited high transcript abundance under multiple conditions. Contrary to expectation, diseased or wounded samples did not show the highest proportion of highly expressed chitinases.

Three of the eight white spruce chitinases associated with autumnal acclimation had a putative Norway spruce orthologue with corresponding RNA-Seq data. GQ03803_J20.1 (class IV) and GQ3902_B01.1 (class II) were putatively orthologous to MA_159244g0020 and MA_10313114g0010, respectively. Both of these genes showed very high transcript abundance in early season developing buds. The former was not expressed more than average in any other tissue, while the latter showed moderately elevated transcript abundance in needles from girdled twigs and needles from vegetative shoots produced in 2010. GQ0207_E09.1 was putatively orthologous to MA_10427109g0010. This sequence showed high transcript abundance in late wood and moderately elevated transcript abundance in early morning needles and pineapple galls.

Twelve additional Norway spruce chitinases, mostly cluster 1/class VII, showed high transcript abundance in tissues associated with the activity-dormancy transition, namely early season buds, late season buds, and late wood. Three of these had a putative white spruce orthologue: Pa_MA_10431378g0020–GQ03811_E13.2, Pa_MA_18470g0010–WS00749_F20.1, and Pa_MA_10262473g0010–GQ03407_A15.1. Pa_MA_10431378g0020 was highly expressed in late buds, while Pa_MA_18470g0010 and Pa_MA_10262473g0010 were highly expressed in late wood.

Discussion

The spruce chitinase gene family

A total of 31 unique white spruce chitinase sequences were identified in this study (Rigault et al. 2011). Eighty distinct partial or full-length Norway spruce chitinases were identified in the v1.0 draft genome sequence (Nystedt et al. 2013), of which 53 were supported by corresponding transcripts. Given the incomplete status of the Norway spruce genome assembly, there is a high likelihood that (a) true genes were undetected, and (b) some sequences represent the same locus, although great care was taken to remove redundant sequences. As such, the number of predicted Norway spruce genes reported here should be viewed as an estimate at best and subject to revision with future versions of the Norway spruce genome. The Norway spruce results imply that the white spruce sequences identified in this study represent only a subset of the total

number of chitinase genes in the white spruce genome. Mining of the v1.0 draft of the white spruce genome sequence (Birol et al. 2013) yielded fewer putative genes than expressed sequences (J. Cooke and K. Muirhead, unpublished), but this will undoubtedly improve with future versions. Notwithstanding, the available evidence from these white spruce and Norway spruce genomic resources suggest that the chitinase family in *Picea* species is expanded relative to that of *Arabidopsis* and rice, which comprise 24 and 37 members, respectively (Xu et al. 2007).

The phylogenetic tree constructed from (a) deduced amino acid sequences of white spruce, Norway spruce, and selected angiosperm species or from (b) deduced amino acid sequences of white spruce showed five well-supported clusters. Xu et al. (2007) identified six distinct clusters for a phylogeny based mainly on the *Arabidopsis* and rice chitinases. However, one of the clusters identified by Xu et al. comprised only three rice chitinases, which were not included in this present analysis. The study by Xu et al. (2007) showed that of the GH 19 chitinases, *Arabidopsis* sequences of class I and II made up one cluster and those of class IV formed a separate sister group, similar to our findings. In our study, class I and most class II chitinases fall into one cluster, while class IV and class VII (Neuhaus 1999) chitinases fall into another cluster. Earlier chitinase classification schemes did not include classes VI and VII (e.g., Hamel et al. 1997). Under the convention of these schemes, chitinases that we have designated as class VII would have been classified as class II. Hamel et al. (1997) argued that class II chitinases behave as an artificial group because (a) the domain structure is inconsistent among this group, and (b) they share several critical amino acids with both class I and class IV chitinases (Hamel et al. 1997; Tyler et al. 2010; Islam et al. 2010). Class VII was designated to accommodate this artificiality (Neuhaus 1999). Consistent with our classification, *P. glauca* × *engelmannii* and *P. contorta* chitinases classified by Kolosova et al. (2014) as class VII fall into cluster 1, show high sequence similarity within the catalytic domain to class IV chitinases, and lack a CBD. These sequences reported by Kolosova et al. (2014) showed high sequence similarity to Pg03803_J20.1 and Pg_GQ0207_E09.1, which we have designated as class VII. Furthermore, although an additional class VII was proposed for cotton chitinases (Li and Liu 2003), and we see that several other sequences form a distinctive cluster with the cotton chitinases (cluster 3), we believe that the classification of these enzymes is doubtful. Li and Liu (2003) proposed that the class VII differ from class II in the presence of a NYNYG domain. We found that this domain is present in several class II chitinases which lie outside this cluster (data not shown). Therefore, while these may be a distinct class of chitinases (or chitinase-like proteins), the same process of domain evolution would allow classification of these sequences as class II and not class VII.

Our study supported the notion that cluster 2/class I and cluster 2/class II chitinases likely have a common ancestor, whereas cluster 1/class VII and cluster 1/class IV chitinases share a different common ancestor (Araki and Torikata 1995; Neuhaus 1999). One of the major discriminating features between class I and class II chitinases is the absence of a CBD in the latter; similarly, the absence of a CBD discriminates class VII from class IV chitinases (Neuhaus 1999). Neuhaus (1999) suggested that the CBD has been lost multiple times during the evolutionary history of the GH 19 family; this notion is supported by the fact that other ancestral lysozymes exhibit the CBD (Hamel et al. 1997). The phylogenetic analysis presented in this study is consistent with this hypothesis. The analysis further suggests that the loss of the CBD in the class VII and class II conifer chitinases occurred following the split between angiosperms and gymnosperms, since the class IV conifer sequences are more closely related to the class VII conifer sequences than to the class IV angiosperm sequences, and the conifer class I and class II chitinases are more closely related to each other than each is to the analogous class of angiosperm chitinases.

We demonstrated that the CBD represents an IDR within the chitinases bearing this domain. IDRs are structurally pliable regions that do not assume a single, stable 3D structure but rather undergo a disorder-to-order transition upon binding of a partner molecule (Tomba 2012; Sun et al. 2013). We expect that the CBD of CBD-containing chitinases with chitinolytic activity recognizes chitin. In contrast, we predict that the CBD of CBD-containing chitinases without chitinolytic activity has other binding partners. IDRs often have the ability to interact with multiple partners, undergoing binding-induced folding to facilitate binding of these different partners (Sun et al. 2013). This raises the intriguing possibility that chitinase CBDs may interact with more than one target substrate or interacts with a given substrate in different configurations.

Putative functions of dormancy-associated white spruce chitinases

Defense against pathogens and pests

Expression of defense-associated genes during overwintering preparation including chitinases has been well-documented in conifers (Ekramoddoullah et al. 1995, 2000; Zamani et al. 2003; Galindo-González et al. 2012). Pathogen-responsive chitinases often belong to classes I and IV (e.g., Sela-Buurlage et al. 1993; Davis et al. 2002; Passarinho and de Vries 2002; Hietala et al. 2004; Islam et al. 2010; Kolosova et al. 2014) and generally exhibit diagnostic active site residues (Holm and Sander 1994; Hart et al. 1995), CBDs (Iseli et al. 1993), and a signal peptide targeting the proteins to the secretory pathway (Nielsen et al. 1997).

Two cluster 2/class I chitinases (Pg_GQ0183_A06.2 and Pg_GQ03904_P01.1) and two cluster 1/class IV chitinases (Pg_GQ03206_D15.1 and Pg_GQ03803_J20.1) exhibited hallmarks of functional chitinases (Table 2). Each of these sequences exhibited three amino acids—Glu, Glu, and Ser (Fig. 6)—demonstrated to be important for catalytic function (Holm and Sander 1994; Hart et al. 1995). However, only Pg_GQ0183_A06.2 and Pg_GQ03904_P01.1 exhibited the characteristic HETT motif of functional chitinases (Passarinho and de Vries 2002), while Pg_GQ03206_D15.1 and Pg_GQ03803_J20.1 exhibited HETG in place of HETT. Recently, Kolosova et al. (2014) demonstrated that a *P. glauca* × *engelmannii* (interior spruce) class I chitinase cDNA nearly identical to Pg_GQ0183_A06.2 and very similar to Pg_GQ03904_P01.1 encoded a protein with chitinolytic activity. In the same study, proteins encoded by *P. glauca* × *engelmannii* class II and class IV chitinase cDNAs nearly identical to Pg_GQ03904_P01.1 and Pg_GQ03206_D15.1, respectively, as well as several cluster 1/class VII *P. glauca* × *engelmannii* and *Pinus contorta* (lodgepole pine) chitinase cDNAs did not display chitinolytic activity (Kolosova et al. 2014). Davis et al. (2002) also reported class II *Pinus elliottii* (slash pine) and *Pinus taeda* (loblolly pine) chitinases that lacked chitinolytic activity.

Kolosova et al. (2014) demonstrated that while only class I chitinases exhibited measureable chitinolytic activity, class I, class II, and class IV interior spruce chitinases were upregulated in response to fungal pathogens (*Leptographium abietinum*) and weevils (*Pissodes strobi*). As such, we infer that the three white spruce chitinases showing the highest similarity to these interior spruce chitinases—Pg_GQ0183_A06.2, Pg_GQ03904_P01.1, and Pg_GQ03206_D15.1—could also play a role in tree defense (Table 2). Each of these three genes was substantially upregulated at the level of transcript abundance during the latter stages of the transition to dormancy, suggesting that these chitinases constitute an important component of the tree's protective arsenal against pests and pathogens that continue to pose a threat to the tree in the autumn and early spring.

Pg_GQ0183_A06.2 and Pg_GQ03904_P01.1 exhibited the CTE shown to be necessary and sufficient for targeting of a tobacco chitinase and a barley lectin to the vacuole (Neuhaus et al. 1991; Dombrowski et al. 1993). We speculate that vacuolar-localized chitinases with chitinolytic activity may act later in the response of the plant to invasion by fungal pathogens or herbivorous insects, when the cell has been disrupted and the contents of the vacuole make contact with the pest or pathogen (Wang and Dong 2011). This model has been proposed for bean, where accumulation of chitinase in the vacuole of leaf cells will have the most effect on the invading fungus when the cell is broken (Mauch and Staehelin 2013). In contrast, it is less clear what functional role the

Table 2 Summary of expression patterns, deduced protein product characteristics, and inferred potential functions of eight dormancy-associated white spruce chitinase genes

Chitinase	Cluster	Class	Domains		Loop 1		No. predicted active site and substrate binding amino acids	OSCs	Relative transcript abundance	Expression profile				Inferred functions		VSP	Cell wall modification	
			SP	CBD	GH	CTE				HETT motif	ST	N	S	R	Defense			Freeze acclimation
Pg_GQ03206_D15.1	1	IV	Y	Y	Y	N	N	6	Y	High	+	+	+	ns	Non-chitinolytic	Y	y	
Pg_GQ03803_J20.1	1	IV	Y	Y	Y	N	N	6	Y	Low	ns	ns	ns	ns		y		
Pg_GQ0183_A06.2	2	I	Y	Y	Y	Y	Y	6	N	Moderate	+	+	+	ns	Chitinolytic		Y	
Pg_GQ03904_P01.1	2	I	N	N	Y	Y	Y	6	Y	High	+	+	+	+	Chitinolytic	Y	Y	
Pg_GQ0207_E09.1	1	VII	Y	N	Y	N	N	5	Y	Low	ns	ns	-/+	+/-				
Pg_GQ03902_B01.1	2	II	Y	N	Y	N	N	5	Y	Low	ns	ns	ns	ns		y		
Pg_GQ03703_O09.1	2	II	Y	N	Y	N	Y	4	Y	High	+	+	+	+	Y	Y		
Pg_GQ0046_J03.1	3	II	Y	N	Y	N	N	4	N	Moderate	-	-	-	-			Y	

For domain analysis, Y indicates presence of the domain, while N indicates absence of the domain. For expression profiles, a plus sign indicates significant upregulation, a minus sign indicates significant downregulation, and ns indicates that changes in transcript abundance were not significant. For inferred function, Y indicates strong evidence, while y indicates circumstantial or weak evidence. SP signal peptide, CBD chitin binding domain, GH glycosyl hydrolase catalytic domain, CTE carboxy-terminal extension.

putatively non-chitinolytic Pg_GQ03206_D15.1 plays in defense.

Wiweger et al. (2003) and Hietala et al. (2004) provided evidence that class IV chitinases are involved in developmental events such as programmed cell death and suggested that class IV chitinases with enzymatic activity that are localized to the apoplastic space may also be involved in signalling through generation of *N*-acetylglucosamine and/or oligonucleotides that in turn trigger changes in gene expression. If this is the case, it is conceivable that the dormancy-associated class IV chitinases identified in this study could be involved in mediating developmental events associated with the activity-dormancy transition.

Freezing acclimation

Freezing tolerance for forest trees and other perennials increases over the course of bud formation and the transition to dormancy (Hawkins and Shewan 2000; Colombo et al. 2003). Chitinases from several species have been shown to play a role in cold and freezing acclimation, with some of these having demonstrated antifreeze activity (e.g., Griffith et al. 1997; Yeh et al. 2000; Pihakaski-Maunsbach et al. 2001; Kikuchi and Masuda 2009; Jarzabek et al. 2009). Chitinases with demonstrated antifreeze activity from *V. corymbosum* (class II) and *S. cereale* (class I and II) are present in cluster 2 (Yeh et al. 2000; Kikuchi and Masuda 2009). Cluster 2 also contains PgGQ0183_A06.2 (class I), Pg_GQ03904_P01.1 (class I), Pg_GQ03703_O09.1 (class II), and Pg_GQ03902_B01.1 (class II). Several lines of evidence suggest that some of these cluster 2 white spruce chitinases play a role in freezing acclimation. Putative ice-binding surfaces were predicted in Pg_GQ03902_B01.1, Pg_GQ03904_P01.1, and Pg_GQ03703_O09.1. Pg_GQ03904_P01.1 and Pg_GQ03703_O09.1 showed strong upregulation during the latter stages of transition to dormancy, particularly following completion of bud formation and the subsequent transfer to low temperatures (4 °C). It is likely that Pg_GQ03902_B01.1 and Pg_GQ03703_O09.1 do not exhibit chitinolytic activity, as neither contained a CBD nor the full complement of putative active site and substrate binding amino acids (Table 2). Pg_GQ03904_P01.1 was truncated at the 5' end, so the presence of a CBD cannot be determined, but the sequence did possess all of the putative active site and substrate binding amino acids. Taken together, these data suggest that Pg_GQ03904_P01.1 (cluster 2/class I) and Pg_GQ03703_O09.1 (cluster 2/class II) may contribute to freezing acclimation in shoot tips, needles, stems, and roots through antifreeze activity.

While most previously characterized chitinases from cluster 1/class IV have been implicated in embryogenesis (Dong and Dunstan 1997; Passarinho et al. 2001; Wiweger et al. 2003) and pest or pathogen responses (Stein et al. 2006;

Zheng et al. 2006; Little et al. 2007; Pré et al. 2008; Islam et al. 2010), some cluster 1/class IV chitinases have been implicated in abiotic stress responses, including cold acclimation (Gong et al. 2001; Oono et al. 2006; Swindell 2006). The cluster 1/class IV chitinase Pg_GQ03206_D15.1, which was strongly upregulated during the latter stages of the activity-dormancy transition, exhibited potential ice-binding surfaces, suggesting that it could function as an antifreeze protein (Table 2).

While the cluster 2/class II Pg_GQ03902_B01.1 and the cluster 1/class IV chitinase Pg_GQ03803_J20.1 have attributes such as putative ice-binding surfaces, the lower and relatively constitutive level of expression of these genes suggests that the proteins play lesser roles in freezing acclimation. It is possible that Pg_GQ03902_B01.1 and Pg_GQ03803_J20.1 require sub-zero temperatures for upregulation and that these chitinases act in complementary timeframes during winter (Hon et al. 1995; Yeh et al. 2000).

It is notable that predicted amino acids bearing the OSCs involved in ice binding map to three distinct regions of the aligned protein sequences (Online Resource 9). It would be interesting to investigate if mutagenesis of such amino acids can influence ice-binding surfaces, once the antifreeze properties have been tested for these chitinases.

Nitrogen storage and seasonal nitrogen cycling

Perennials undergo seasonal nitrogen cycling, whereby nitrogen resources such as vegetative storage proteins (VSPs) are accumulated in perennating tissues as the growing season ends, and remobilized as needed to sustain metabolic and biosynthetic activities during the winter months and the initial phases of regrowth the following spring (Cooke and Weih 2005). VSP accumulation during the late summer and early autumn has been characterized for a number of perennials, including forest trees (Wetzel et al. 1989; Langheinrich and Tischner 1991; Avice et al. 2003). Proteins from diverse families have been recruited to function as VSPs, including chitinases. Although most chitinases characterized as VSPs are GH 19 cluster 4/class III chitinases (Peumans et al. 2002; Meuriot et al. 2004; Rao and Gowda 2008), a storage function for chitinases belonging to other classes cannot be discounted. There is also the possibility of multi-functional chitinases, such as chitinolytic chitinases that serve as VSPs (Meuriot et al. 2004) or chitinolytic chitinases with additional VSP and cold acclimation functions (Avice et al. 2003). Chitinolytic chitinases could conceivably even contribute to seasonal N cycling by releasing nitrogen from *N*-acetylglucosamine-containing endogenous substrates, although this is speculative.

Roberts et al. (1991) and Binnie et al. (1994) demonstrated that *P. glauca* × *Picea engelmannii* accumulated proteins during fall acclimation and that these proteins likely functioned as VSPs. The identity of these proteins was not established,

however. The protein corresponding to cluster 2/class I Pg_GQ0183_A06.2 (cluster 2/class I) showed the largest accumulation of all proteins during the activity-dormancy transition (Galindo-González et al. 2012) and thus is also a candidate VSP. The pattern of transcript abundance for this sequence is in agreement with the protein accumulation pattern. Three other autumn-associated chitinases, Pg_GQ03703_O09.1 (cluster 2/class II), Pg_GQ3904_P01.1 (cluster 2/class I), and Pg_GQ03206_D15.1 (cluster 1/class IV), also showed substantial increases in transcript abundance during the activity-dormancy transition.

VSPs are often localized to vacuoles for sequestration (Wetzel et al. 1991; Sauter and van Cleve 1994). Galindo-González et al. (2012) showed that white spruce stems accumulate considerable protein in storage vacuole-like bodies in the phloem parenchyma and ray cells during the transition to dormancy, similar to VSP accumulation in other species. The CTE present on the cluster 2/class I chitinases Pg_GQ03904_P01.1 and Pg_GQ0183_A06.2 suggests that these proteins could be targeted to the vacuole, providing further evidence that these chitinases are VSP candidates. Further experiments to determine the annual pattern of protein abundance and subcellular localization are necessary to ascertain if these chitinases function as VSPs.

Interestingly, the three class III white spruce chitinases identified in this study all showed greatest expression in megagametophytes and embryogenic tissue, raising the possibility that they serve a storage role in these tissues even though they are not classical seed storage proteins.

Cell wall modification

Pg_GQ0046_J03.1 grouped with class II *Arabidopsis* CTLs shown to be involved in cell wall modification (Zhang et al. 2004; Sánchez-Rodríguez et al. 2012) in cluster 3. Sánchez-Rodríguez et al. (2012) demonstrated that AtCTL1 and AtCTL2 affect cellulose biosynthesis, possibly by promoting interactions between cellulose microfibrils and matrix glycans. Pg_GQ0046_J03.1 exhibited a signal peptide but lacked a CTE, suggesting that it is targeted to the cell wall (Table 2). Pg_GQ0046_J03.1, which was shown by digital expression profiling to be very highly expressed in all tissues with extensive cell wall biosynthesis activity, was the only one of the eight dormancy-associated chitinases that was downregulated during the activity-dormancy transition. The timing of downregulation mirrored growth cessation and the concomitant completion of cell maturation and showed similarities to transcript profiles of other known cell wall biosynthetic genes (El Kayal et al. 2011; Galindo-González et al. 2012). Furthermore, Pg_GQ0046_J03.1 lacked one of the conserved substrate binding amino acids and did not have a CBD, hinting that the preferred substrate for this protein is not chitin but perhaps another carbohydrate such as the glucan polymers

that are bound by AtCTL1 and AtCTL2. Preliminary experiments indicate that, indeed, the protein coded for by Pg_GQ0046_J03.1 does not hydrolyze chitin in *in vitro* assays (El Kayal, Morris, and Cooke, unpublished).

Conclusion

In this study, we have demonstrated that a diverse array of chitinases participate in the transition of white spruce from active growth to dormancy, playing putative roles in defense, cold acclimation, seasonal nitrogen cycling, and cell wall modification. *In silico* analyses suggest that some of these dormancy-associated chitinases are multifunctional. No two chitinases appear to be redundant in their spatiotemporal expression pattern and inferred function. In some cases, closely related chitinases exhibit amino acid differences that lead to different functions, suggestive of neofunctionalization. In other cases, closely related chitinases with very similar sequences exhibit different patterns of transcript abundance across tissues over time, suggestive of subfunctionalization (Duarte et al. 2006; Lynch and Conery 2012). Tyler et al. (2010) hypothesized that because of a higher rate of mutation in the catalytic domain of GH 19 members than that of GH 18 members, the former exhibit adaptive functional modifications that emerge as a result of pathogen pressure and therefore increased genetic variability that could result in functional diversity. In support of this notion, Liu et al. (2011) recently demonstrated that nucleotide variation in a class IV chitinase gene was significantly associated with *Pinus monticola* resistance against the pathogen *Cronartium ribicola*. SNPs have been identified and validated for most of the white spruce chitinases reported in this study (Pavy et al. 2013). It will be of great interest to determine if non-synonymous SNPs or indels observed within populations can be connected to altered chitinase functional attributes and if there is any relationship between these altered functional attributes and signatures of selection.

In summary, our results imply that through a non-redundant complement of spatiotemporal expression patterns and functions, this suite of chitinases contribute to the tree's ability to withstand the rigors of winter and resume growth the following spring. In-depth functional studies of these chitinases are now underway to determine whether the *in vitro* and *in vivo* functions of these proteins are consistent with the functions predicted by *in silico* analysis. Availability of the draft white spruce genome sequence (Birol et al. 2013) facilitates analysis of the regulation of these genes through promoter studies.

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Data archiving statement NCBI GenBank accession numbers for all white spruce sequences are provided in Online Resource 1.

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