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ORIGINAL ARTICLE

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Characterization of Picg5 novel proteins associated with seasonal cold acclimation of white spruce (*Picea glauca*)

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Abstract Four protein bands of 38, 34, 32 and 25 kDa (designated as Picg5a to Picg5d) were detected specifically with an antibody against a putative PR10 protein Picg1 in *Picea glauca*. The Picg5 proteins accumulated during cold acclimation in foliage and roots and showed polymorphism in the population. Only one Picg5 protein was expressed in each individual seedling. The Picg5 protein levels were positively correlated with freezing tolerance (R^2=0.6, P<0.001). Two types of cDNA clones (designated as *Picg5-1* and *Picg5-2*) were characterized after screening an expressed cDNA library with anti-Picg1 antibody. Compared to *Picg5-1*, *Picg5-2* has a deletion of 123 nucleotides in the coding region. *Picg5-1* and *Picg5-2* shared 97–98% identity on the nucleotide and deduced amino acid sequence levels. Southern blot analysis suggested one or two copies of the *Picg5* gene within the *P. glauca* genome. Recombinant proteins by expressing *Picg5* cDNAs in *Escherichia coli* had the same antigenic binding property and molecular masses of natural proteins, suggesting that they encode Picg5 proteins. The amino acid sequence deduced from *Picg5* cDNAs consisted of five or six copy repeat segments (termed QKA segments). A BLASTP search of GenBank data revealed that the Picg5 proteins were novel ones, only two QKA segments showing low similarity with the K-segment of both dehydrin and rehydrin. Like protein accumulation, the *Picg5* transcripts were up-regulated during cold acclimation. The expression pattern and unique features of protein structure suggest that the Picg5 proteins may have a function related to cold stress.

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Introduction

Many perennial plant species in temperate regions go through an annual cycle of a growing phase in summer and a dormant phase in winter. Plants undergo adaptive changes in response to environmental clues including low nonfreezing temperatures and shorter photoperiods (Sakai and Larcher 1987; Huner et al. 1998; Zwiazek et al. 2001). This process, called cold acclimation, allows hardy plants to obtain efficient frost tolerance mechanisms needed for winter survival (Levitt 1980). A series of morphological, physiological, and biochemical changes occur in plant cells during cold acclimation (Guy 1990; Thomashow 1999; Zwiazek et al. 2001). These changes include altered solution components in apoplastic space (Griffith et al. 1997), enhanced rigidity of cell walls (Rajasheker and Lafta 1996), varied lipid composition and properties of plasma membranes and the membrane systems inside cells (Steponkus 1984; Uemura et al. 1995), and increased concentration of compatible osmolytes in the intracellular environment (Guy 1990; Hare et al. 1998).

During overwintering, plants encounter freezing stress that results in the desiccation of cell protoplasm. Under freezing temperatures, the ice formed initially in the intercellular space causes water to move out of the cell across the plasma membrane (Thomashow 1999). This type of freeze-induced dehydration produces multiple injury effects to plant cells, including denaturation of proteins, increased concentration of intracellular solutes, precipitation of different molecules, and various forms of membrane damage (Steponkus 1984; Steponkus et al. 1998). Freeze-induced damage to cell membrane systems is considered the primary injury in plants resulting from severe dehydration under freezing temperatures (Steponkus 1984). Tolerance to dehydration, therefore, is a key factor for plant cell survival during freezing stress.

As a qualitative trait, cold tolerance is believed to be associated with hundreds of genes or proteins. Since the first report on gene expression change in spinach under chilling temperatures (Guy et al. 1985), more than 100 genes have been reported to be involved in cold acclimation (Thomashow 1999). According to the properties of their protein products, these genes encode protein components of cold signal transduction, antifreeze proteins, or enzymes associated with increased freezing tolerance (Guy 1990; Urrutia et al. 1992; Hughes and Dunn 1996; Griffith et al. 1997; Gilmour et al. 1998; Thomashow 1999). Many other proteins are proposed to stabilize subcellular architecture under dehydration stress, such as late-embryogenesis abundant proteins (LEAs), dehydrins, and heat-shock proteins (HSPs) (Neven et al. 1992; Ingram and Bartels 1996; Close 1997; Steponkus et al. 1998). However, the precise role these genes or proteins play in the process of cold acclimation remains to be elucidated.

Despite the fact that physiological studies of cold acclimation began in woody plants (Weiser 1970; Burke et al. 1976), knowledge of cold acclimation at the molecular level has been derived mainly from the study of herbaceous plants. As for many crop plants, freezing is the most severe limiting factor for forest production in temperate regions. White spruce (*Picea glauca*), a very important timber species, is one of the most widely distributed and important wood-producing conifers in North America. Reforestation of white spruce has been difficult in many regions because of freezing-related injuries in nursery-generated seedlings during their first winter (Krasowski et al. 1996). Therefore, identification of cold-induced proteins/genes for frost hardiness in white spruce or other conifers would meet both economic and basic research interests and constitute a key step toward the understanding of freezing injury and tolerance mechanisms. Such knowledge could also provide transgenic tools or molecular markers for improvement of freezing tolerance in white spruce.

In our earlier studies, protein profiles in *Pinus* species over a period of natural cold acclimation were investigated using 2-D SDS-PAGE analysis (Ekramoddoullah et al. 1995; Ekramoddoullah and Taylor 1996). A novel protein (Pin m III) was revealed with seasonal regulation in western white pine (*Pinus monticola*). An electrolyte leakage analysis established a significant correlation between Pin m III level and frost hardiness during seasonal cold acclimation (Ekramoddoullah et al. 1995). Protein sequence analysis and cDNA cloning demonstrate that Pin m III belongs to a super family of class 10 pathogenesis-related proteins (PR10) (Yu et al. 2000). Multiple PR10 proteins and their corresponding genes have been identified in western white pine and they are differentially accumulated in response to cold hardiness, wound and fungal infection (Liu et al. 2003). A PR10 protein also displays a seasonal expression pattern in Douglas-fir (*Pseudotsuga menziesii*) (Ekramoddoullah et al. 2001). Some PR proteins, such as chitinases, β-1, 3-glucanases, and thaumatin-like proteins, have been reported to be bifunctional, i.e., anti-fungal and anti-freeze (Griffith et al. 1997).

Pin m III homologous PR10 cDNAs (*Picg1*, *Picg2*, and *Picg4*) were isolated from white spruce recently (Matheus et al. 2003). In the present work, novel proteins of Picg5a–d were serendipitously found because of their specific immunoreaction with the anti-Picg1 antibody raised against an antigenic region of the putative protein encoded by PR10 *Picg1*. We investigated seasonal regulation of Picg5 proteins and found that a high level of protein accumulation was correlated with the process of seasonal cold acclimation in both root and foliage tissues. To gain new insights on the role of these proteins in frost hardiness, we isolated and characterized cDNAs encoding for Picg5 proteins.

Materials and methods

Plant material

White spruce seedlings of seed lot #31310 were grown under natural conditions. For northern blot analysis, total RNA was isolated from pooled samples of multiple seedlings collected from July to November 2000 from the field at Colt Creek, BC, Canada. For western immunoblot analysis to study seasonal regulation of Picg5 proteins, total protein was extracted from plant samples collected monthly from December 1993 to January 1995 at the Pacific Forestry Centre, Victoria, BC, Canada. All samples were flash frozen in liquid nitrogen for subsequent storage at −80°C until use.

Protein extraction and western immunoblot analysis

Total protein was extracted from frozen plant tissues pooled from more than 100 individual seedlings and protein concentration was determined using BSA as a standard (Ekramoddoullah and Davidson 1995). In our search for the expression of a putative protein from cDNA *Picg1*, a rabbit anti-Picg1 antibody was raised against an antigenic region (LPGAPDGETRVEEIKKMDD) of this hypothetical protein (Matheus et al. 2003). The synthesis of the peptide, subsequent production and purification of the anti-peptide antibody, and western immunoblot analysis were carried out as described before (Ekramoddoullah et al. 1995). Scanning and quantitation of the western blots were performed with BIORAD Quantity One software (Version 4.2.3, Bio-Rad Laboratories, Hercules, Calif., USA).

Assessment of frost hardiness and expression level of Picg5 proteins

Correlation analyses of freezing tolerance and Picg5 protein levels were performed as described previously (Ekramoddoullah et al. 1995). Eighty seedlings were

raised at University of Victoria, BC. Foliage of 10 individual seedlings was harvested eight times from July to December 2002 for the quantification of Picg5 proteins with western blot analysis and assessment of freezing tolerance with electrolyte leakage analysis. Because of the high freezing tolerance of this seed lot, the temperature resulting in 10% damage to samples was interpolated from the index of injury at three freezing temperatures. The correlation between frost hardiness and Picg5 protein level was examined by linear regression analysis.

Immunoscreening of a cDNA library and cDNA cloning

A cDNA library was constructed in λ Uni-ZAP XR vector (Stratagene, LaJolla, Calif., USA) from mRNAs of roots pooled from a few cold-hardened seedlings of seed lot #31310 (Matheus et al. 2003). The cDNA library was screened with a picoBlue Immunoscreening Kit (Stratagene) following the procedure as recommended by the manufacturer. Thirty-one positive clones were found from the screen of 1×10^6 clones of the amplified cDNA library. Positive lambda clones were purified with three to four rounds of screening. Following in vivo excision of positive plaques, all plasmids were isolated using a MiniPrep plasmid purification kit (Qiagen).

The reaction of rapid amplification of cDNA 5′-end (5′-RACE) was performed to confirm the full-length coding region of the isolated cDNA clones. The first strand cDNA was synthesized using a SMART cDNA library construction kit (Clontech Laboratories, Palo Alto, Calif., USA) following the manufacturer's instructions. For cDNA amplification, a gene-specific antisense primer, 5′-AAG CAC TCT GAG ATT GAG TGT TGG-3′, was used together with 5′-PCR primer provided by the manufacturer (Clontech Laboratories). Thirty cycles were performed at 94°C for 30 s and at 68°C for 2 min. The 5′-RACE product was cloned into pGEM-T easy vector (Promega, Madison, Wis., USA) and sequenced.

DNA sequence analysis and nucleic acid hybridization

The complete nucleotide sequences of both strands of full-length cDNA inserts were determined on an ABI310 DNA sequencer (Applied Biosystems) using a Thermo-cycle sequence kit (Amersham) at the University of Victoria, BC, Canada. DNA sequence data were assembled and analyzed using BLAST network services at the National Center for Biotechnology Information (NCBI) (Altschul et al. 1997). The sequence alignment and protein structure predictions were performed with the computer software package DNASTAR (DNASTAR, Madison, Wisconsin, USA).

RNA was extracted from tissues pooled from a few white spruce seedlings as described by Matheus et al. (2003). For northern blot analysis, total RNA (10 μg per lane) was electrophoresed in a denaturing formaldehyde agarose gel with RNA molecular mass standards (Life Technologies, Burlington, ON, Canada), blotted onto a Hybond-N+membrane (Amersham-Pharmacia Biotech), and hybridized using a buffer of "DIG easy hyb" (Boehringer Mannheim, Laval, Québec, Canada) following standard procedures as described by Sambrook et al. (1989). A cDNA probe of *Picg5* was labeled with digoxygenin (DIG) using a random primer labeling kit (Boehringer Mannheim). The blots were washed finally twice at 68°C for 30 min with 0.1× SSC and 0.1% SDS and detected with a DIG detection kit (Boehringer Mannheim). The equality of loaded RNA samples was verified by gel staining with ethidium bromide.

Because of the detected polymorphism of Picg5 proteins, genomic DNA from one single white spruce seedling was used for southern blot analysis. Genomic DNA was purified using a Plant DNeasy Extraction Kit (Qiagen), and cut with restriction enzymes as indicated. Digested genomic DNA (20 μg) was separated on 0.8% agarose gel. The blot transfer, hybridization and washing conditions were the same as that for RNA hybridization.

Expression of *Picg5* cDNAs in *E. coli*

cDNA inserts were cloned into the Eco R I/Xho I sites of pBluescript SK (−) vector (Stratagene) and subsequently transformed into host *E. coli* strain SOLR cells. Cells were grown in 100 ml LB medium at 37°C at 220 rpm. When the cultures reached $A_{600\ nm}$=0.6, 10 mM IPTG was added to induce the *lac Z* fusion promoter and cultures were incubated for 2 h more. After collection by centrifugation, cells were resuspended in a buffer containing 50 mM TRIS (pH 8.0), 10 mM EDTA and 5 mM β-mercaptoethanol. The resuspended *E. coli* cells were added with equal volume of 2× SDS-PAGE loading buffer, and treated at 100°C for 3 min before gel running for western immunoblot analysis.

Results

Accumulation of Picg5 proteins during seasonal cold acclimation

Western immunoblot data showed that the anti-Picg1 antibody detected four protein bands with molecular mass of 38, 34, 32 and 25 kDa (designated as Picg5a to Picg5d) in the foliage (Fig. 1). The antibody failed to detect a 16 kDa protein predicted from cDNA *Picg1*, providing further evidence for the previous suspicion of an artifact in *Picg1* cloning (Matheus et al. 2003). The data also showed that Picg5 accumulation was associated with periods of cold acclimation. Picg5 proteins were found to be abundant in December, January and February, low in March and April, and the protein decreased to an undetectable level in May, June, July, and August (Fig. 1). Picg5 became detectable in September again, and reached a high level in November (Fig. 1). A similar

pattern of Picg5 accumulation was also observed in the root tissues of the same seed lot and in the foliage of two other seed lots (data not shown). Total protein samples from seeds and seedling tissues were examined by western immunoblot analysis using the anti-Picg1 antibody during seed germination up to 21 days when the average length of the seedlings was about 50 mm. No immunostained band was detectable in these samples (data not shown).

When protein blots of winter samples from individual seedlings were analyzed by anti-Picg1 antibody, different protein patterns were detected. Only one Picg5 protein was detected as a major band in each seedling (Fig. 2). Minor bands were observed with smaller molecular mass in the seedlings expressing Picg5a or Picg5b (Fig. 2, lanes 2–6). These results suggested a possible protein degradation or post-translational processing for Picg5a and Picg5b. A large proportion of seedlings expressed Picg5b or Picg5d. Only a few seedlings expressed Picg5a as the major band in the tested population. These results indicated a Picg5 protein polymorphism in the population.

Figure 3 shows the Picg5 level and corresponding frost hardiness of white spruce seedlings during a cold acclimation period. There was a significant correlation between protein level and freezing temperature resulting in 10% index of injury (LT_{10}) as determined by electrolyte leakage

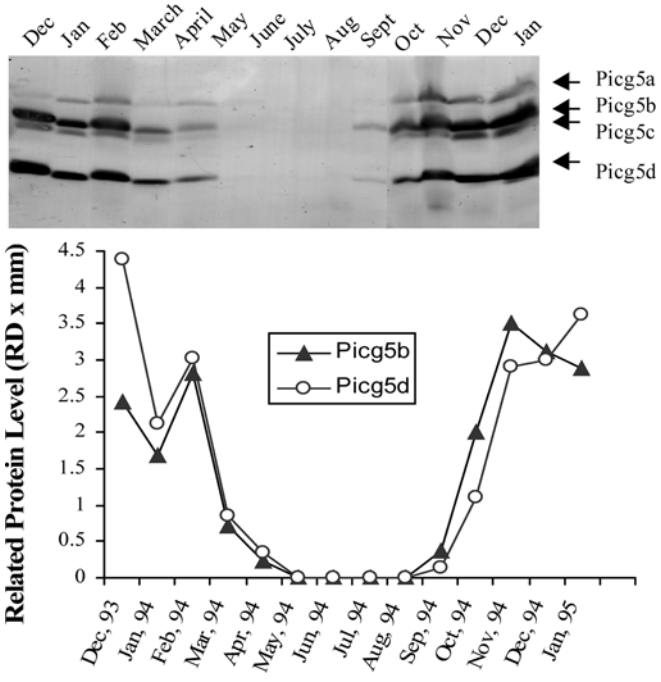

Fig. 1 Immunodetection of the white spruce protein profiles using the anti-Picg1 antibody. (*Top*) Protein profile of foliage tissues from seed lot #31310 from December 1993 to January 1995. Equal amounts of total protein (100 μg) from the pooled tissues of foliage was separated on an SDS-polyacrylamide gel, and transferred to a PVDF membrane. Proteins were detected on the western blots using anti-Picg1 antibody. Individual lanes were loaded with protein samples from the pooled foliage collected from one hundred seedlings on the indicted dates. (*Bottom*) Picg5 protein levels were quantified using PDI software. Similar patterns of Picg5 accumulation were observed in the roots from the same seed lot and foliage from two other seed lots in independent experiments

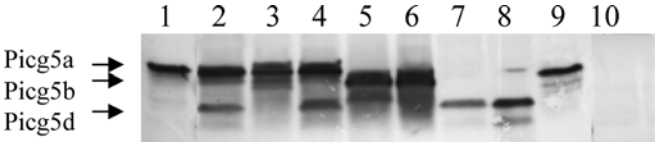

Fig. 2 Polymorphism of Picg5 proteins and *Picg5*cDNA expression in *E. coli*. Protein extracts from bacterial strain XL-1 cell lines and from white spruce individual seedlings in the winter season were detected by western immunoblot analysis. After SDS-PAGE separation and transfer to a PVDF membrane, the proteins were detected using anti-Picg1 antibody. *Lane 1*, XL-1 cell lines expressing *Picg5-1*; *lanes 2–4*, Picg5a protein (38 kDa) expressed in three representative seedlings; *lanes 5–6*, Picg5b protein expressed in two representative seedlings; and *lanes 7–8*, Picg5d protein expressed in two representative seedlings; *lane 9*, XL-1 cell lines expressing *Picg5-2a*; lane 10, Xl-1 cell line containing expression vector without cDNA insert

in individual seedlings (R^2=0.6, P<0.001). These results indicated that Picg5 proteins were up-regulated during seasonal cold acclimation and their expression levels were correlated with freezing tolerance during cold acclimation.

Cloning and nucleotide sequence of cDNAs encoding Picg5 proteins

Picg5 proteins were shown to react with the anti-Picg1 antibody (Fig. 1). Therefore, any cDNAs encoding these proteins could be selected by immuno-screening of an expression cDNA library with this antibody. As a result, several positive cDNA clones were identified after cDNA library screening. Among them the cDNA clone *Picg5-1* is longest; no in-frame stop codon is detected upstream from the first ATG (Fig. 4). The context of the first ATG (AGTATGGCT) has 78% identity of a translation initiation site consensus (ACAATGGCT) (Joshi 1987). 5′-RACE was performed to locate a start codon further upstream. Sequence analysis of 5′-RACE fragments

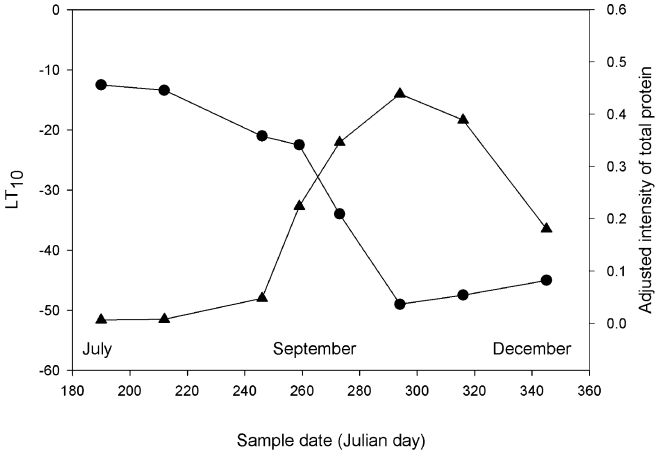

Fig. 3 Seasonal variation in the frost hardiness and the level of Picg5 proteins in white spruce seedlings. Foliage from 80 seedlings was collected at different harvest dates as indicated. The temperature resulting in 10% damage (LT_{10}) (*filled circles*) of needles was assessed by electrolyte leakage analyses and the relative content of Picg5 proteins (*filled triangles*) was evaluated using western immunoblot analyses

showed that no additional nucleotide sequence was found upstream of the first nucleotide of *Picg5-1*, suggesting that its open reading frame (ORF) may indeed be complete.

The full-length sequence of *Picg5-1* is 1,148 bp in length, consists of an ORF of 792 bp flanked by a 5′-untranslated region (UTR) of 67 bp and 3′-UTR of 388 bp. *Picg5*-1 cDNA encodes a putative protein of 263 amino acids with a calculated molecular mass of 28.5 kDa and a pI of 5.53. Another type of cDNA *Picg5-2*, including two different clones *Picg5-2a* and *Picg5-2b*, has a deletion of 123 bp inside the coding region. There is a 7 bp direct repeat (TGCTTCC) at the beginning and end of the deletion, suggesting that a loop-out event might have resulted in the removal of the deleted sequence in *Picg5-2*. Furthermore, *Picg5-2* cDNAs share identities of 97–98% with *Picg5-1* (Fig. 4), including largely identical nucleotide sequence in the 3-UTRs, indicating that *Picg5* cDNAs probably originated from the same genomic locus.

Efforts to perform N-terminal amino acid sequence analysis on the purified Picg5 proteins were not successful possibly due to the blockage of the N-terminal amino acid. The evidence linking cDNA clones *Picg5-1* and *Picg5-2* with Picg5 proteins was provided by cDNA expression in *E. coli*. *Picg5* cDNA inserts were cloned in pBluescript SK (−) under the control of the *lac* promoter. Sequence analysis showed one stop codon in frame in the fusion region for *Picg5-1*, so it was supposed to translate from its own start codon with an expected size of 28.5 kDa. The *Picg5-2a* cDNA was fused in frame with *Lac Z*, and was expected to translate into a fusion protein with a calculated molecular mass of 27 kDa. Western immunoblot analysis showed no immunoreactivity in the negative control of *E. coli* extract possessing expression vector without cDNA inserts (Fig. 2, lane 10), whereas one strong staining band of 38 kDa was revealed in extracts of the bacterial cells expressing *Picg5-1* (Fig. 2, lane 1). The molecular mass

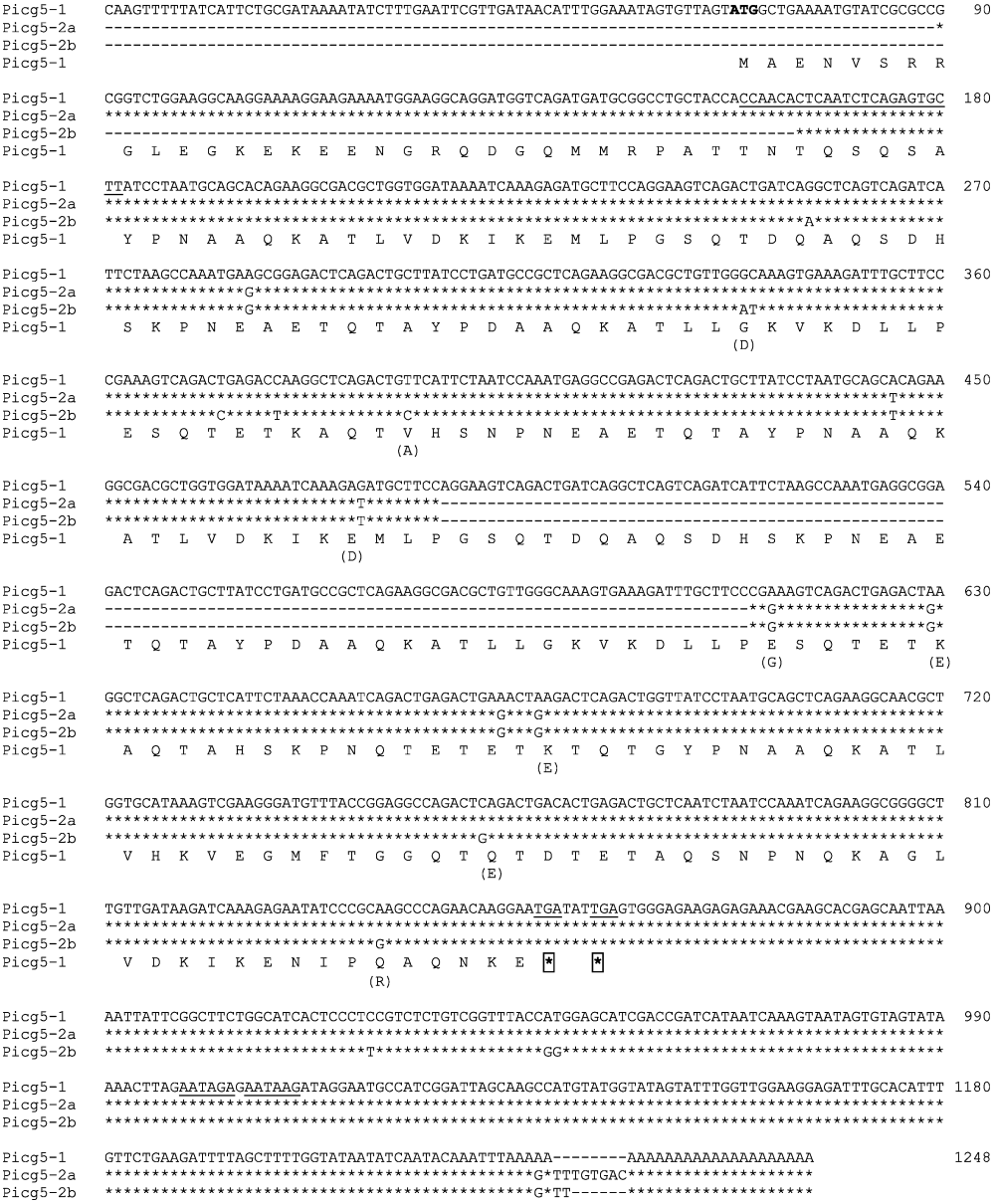

Fig. 4 Comparison of nucleotide sequences and deduced amino acid sequences of white spruce cDNA clones *Picg5-1*, *Picg5-2a*, and *Picg5-2b* (GenBank accession number AY220128–AY220130). Two tandem stop codons at 3′-UTR are represented by *bold asterisk* in box. *Bold letters* indicate the putative start codon. The putative polyadenylation sites and the sequence region for 5′RACE primer are underlined. The nucleotides identical to the first line are indicated by *asterisk*. *Dashes* indicate the deletion or gaps in the sequences. The amino acids different from those predicted from cDNA clone *Picg5-1* are shown in parentheses

and immunoreactivity of the recombinant protein from *E. coli* are the same as that of an authentic Picg5a protein (Fig. 2, lanes 2–4). A band of 34 kDa was detected in bacterial extracts expressing *Picg5-2a* (Fig. 2, lane 9), corresponding to the size of Picg5b from individual seedlings (Fig. 2, lane 5–6). These results suggested that the Picg5a protein may be encoded by *Picg5-1*, and that Picg5b may be encoded by *Picg5-2*. Picg5c and Picg5d may be the processed products of *Picg5-1/Picg5-2* or encoded by other unidentified alleles of *Picg5* gene.

The molecular mass of the *Picg5* products observed on SDS-PAGE blots was different from that predicted from the cDNA sequence. Because of the lack of post-translational processing in *E. coli*, it is not known why there is a discrepancy of protein molecular mass between theoretical calculation and experimental detection. This discrepancy phenomenon has also been reported previously with several cold-induced proteins of angiosperms (Neven et al. 1993; Danyluk et al. 1998; Kaye et al. 1998).

Characteristics of Picg5 protein structure

The protein structure of Picg5a as deduced from cDNA *Picg5-1* has several unusual characteristics that may provide some insights into its biological function. Six imperfect tandem repeats, designated as QKA segments because of the presence of conserved three amino acid residues in them, are detected from residue 33–261 (Fig. 5A). According to *Picg5-2* nucleotide sequence, the fourth QKA segment of Picg5a was deleted in Picg5b and others are almost identical between them. The Picg5a protein is rich in Ala (12%), Thr (12%), Gln (11%), Lys (10%) and Glu (9%), but devoid of both Cys and Trp, which explains its high hydrophilicity (Fig. 6A). The secondary structure of the Picg5a protein is predicted to consist almost completely of α-helix (47.53%) and random coil (49.43%) (Fig. 6B,C). The highly conserved core sequences in each QKA segment, AAQKATLVD-KIKE, are predicted with highest possibility to generate an amphipathic α-helical region (Fig. 6B,D), and the sequences around them are predicted to form random coils (Fig. 6C).

A search of the GenBank database revealed that the Picg5a protein sequence shared highest similarity of 22% with the spinach cold acclimation protein CAP85, a member of the group II LEAs/dehydrin super-family (Neven et al. 1993). This limited homology is restricted to two QKA repeats (repeat II and IV) and the conserved K-segment of LEAs-II/dehydrin (Close 1997) and rehydrin (Velten and Oliver 2001) (Fig. 5B).

Expression of a small family of *Picg5* genes during seasonal cold acclimation

The copy number of *Picg5* genes in the white spruce genome was estimated by southern blot analysis of genomic DNA from one single seedling. Two strong bands were detected following digestion with restriction enzyme Nco I or Nde I (Fig. 7). Because cDNAs of *Picg5-1* and *Picg5-2a* contain one internal Nco I site and no Nde I site, these results suggest that there are only one or two copies of *Picg5* genes in the white spruce genome.

The transcript levels of *Picg5* genes were analyzed in foliage by northern blot hybridization with a *Picg5-1* cDNA probe. During natural cold acclimation from the growing season to the dormant season, mRNAs of *Picg5* genes were initially detected in the middle of August, reached peak expression level in the middle of October, and decreased gradually thereafter (Fig. 8). The two mRNA bands detected on the northern blots were about 1.1 and 1.3 Kb long, which were consistent with different sizes of cDNA clones *Picg5-1* and *Picg5-2*. Although the patterns of transcript accumulation were similar, *Picg5-1* and *Picg5-2* are expressed differentially. Compared with the transcript of *Picg5-1*, the peak level of *Picg5-2* transcript was lower in the middle of October and after that time its levels decreased more rapidly (Fig. 8).

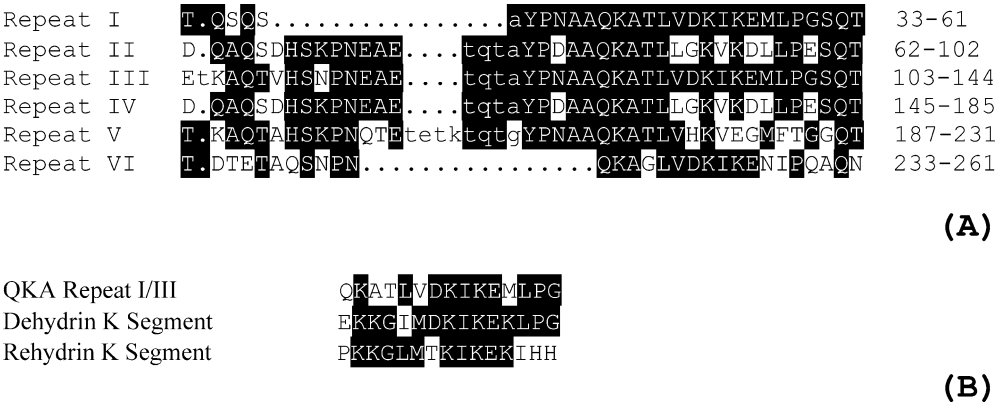

Fig. 5 **A** Alignment of tandem QKA repeat units (I–VI) in the Picg5a protein predicted from the cDNA sequence of *Picg5-1* with software DNASTAR. The fourth repeat was deleted as predicted from *Picg5-2*. The amino acid sequence is numbered at the left. The conserved amino acid residues are represented in the *black*. **B** Comparison of the core sequence of QKA repeats with K-segment consensus of group 2 LEA/dehydrin and rehydrin. Letters boxed in *black box* are the identical amino acid sequences

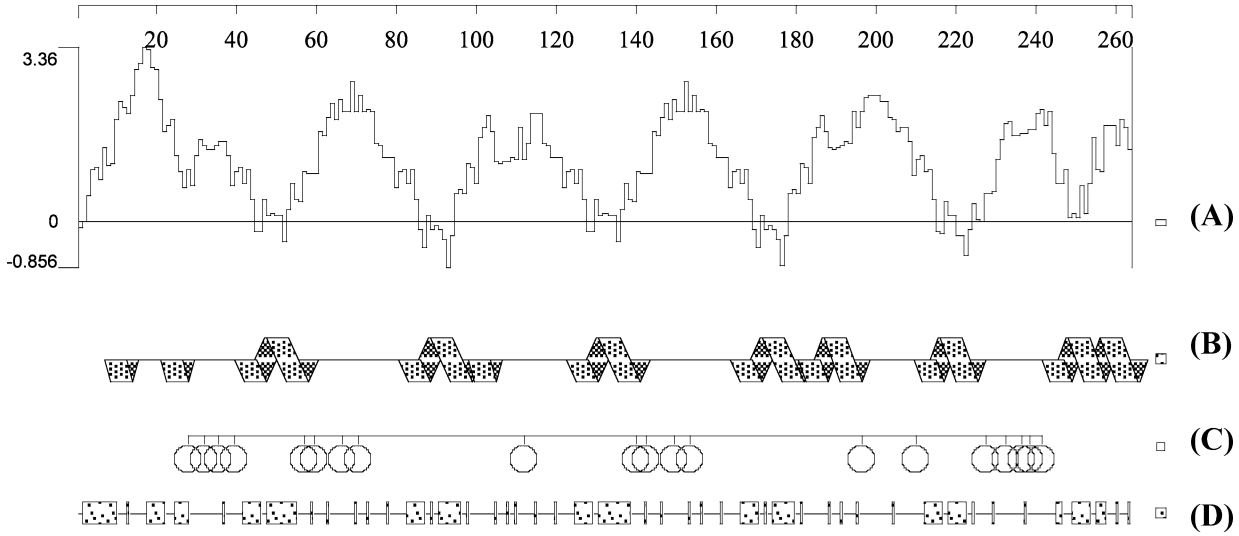

Fig. 6 The diagrammatical representation of properties and secondary structures of the Picg5a protein predicted from cDNA clone *Picg5-1*using software package of DNASTAR. The *top scale* shows the order of amino acid sequence. **A** Hydrophilicity plot using Kyte–Doolittle's method. **B** α-helix regions predicted by Chou–Fasman decisions. **C** Coil regions predicted by Garmier–Robson decisions. **D** α-amphipathic regions predicted by Eisenberg's method. The core sequences of six QKA repeat are predicted to form α-helices **B** with amphipathic property **D**, and the regions flanking QKA core sequence to form random coil structure **C** that corresponds to six hydrophilic peaks **A**

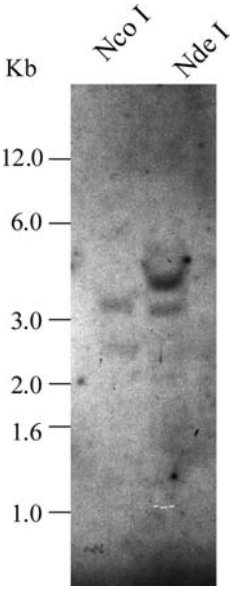

Fig. 7 Southern blot hybridization of white spruce genomic DNA. Genomic DNA (20 μg) was digested with Nco I or Nde I and electrophoresed on a 0.8% agarose gel. The blot was probed with cDNA insert of *Picg5-1*. Molecular sizes are indicated at the *left* in Kb

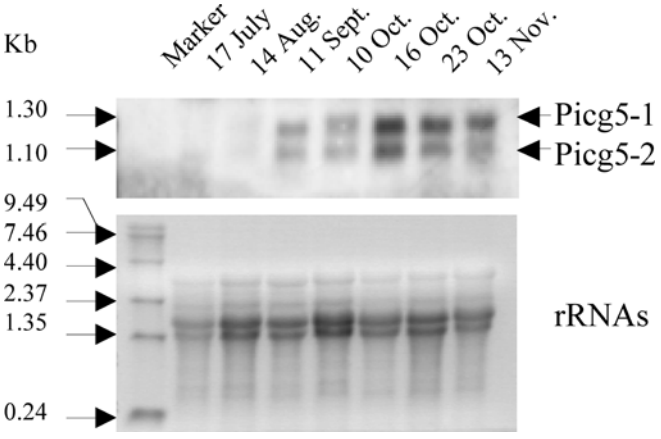

Fig. 8 Expression of *Picg5*mRNAs in foliage during cold acclimation. Total RNA from foliage of seed lot #31310 at different cold acclimation stages was analyzed with northern blot hybridization using an insert of *Picg5-1* cDNA as probe. Equal amounts of total RNA (10 μg) were separated by agarose gel electrophoresis in the presence of formaldehyde and transferred to a nitrocellulose membrane. *Top panel*: X-ray film image of northern blot. The size of *Picg5-1* and *Picg5-2*transcripts is indicated on the right in Kb. *Bottom panel*: equal loading was determined by visualization of rRNAs

Discussion

We report here the characterization and seasonal regulation of white spruce Picg5 proteins during cold acclimation. Western immunoblot analysis clearly indicated that Picg5 proteins were most abundant in winter months followed by a gradual decline in late spring, becoming undetectable in summer months (Fig. 1). The accumulation pattern of Picg5 is similar in roots and foliage and in different seed lots. The content of Picg5 proteins is correlated significantly with the seasonal changes of cold-hardiness in individual seedlings (Fig. 3). Considering the absence of Picg5 in seeds and seedlings during germination, the synthesis of Picg5 is likely to be seasonally regulated in response to environmental stresses such as low temperatures and short day lengths in the fall. Screening of a cDNA library recovered two cDNA clones *Picg5-1* and *Picg5-2* (Fig. 4). Northern blot analysis demonstrated that their mRNA expression was induced with the period of seasonal cold acclimation (Fig. 8), similar to, but ahead of the accumulation of the Picg5 proteins (Fig. 1). These observations suggest that the seasonal accumulation of Picg5 proteins may be regulated at the transcriptional level.

The expression pattern and their correlation with freezing tolerance suggest that the Picg5 proteins probably play an important role during cold acclimation. Because of the continuously and highly accumulated levels during cold acclimation, the Picg5 proteins are unlikely to be a regulatory factor in the development of cold hardiness. The amino acid sequence of Picg5a as deduced from *Picg5-1* cDNAs shows no significant identity to antifreeze or other cold-induced proteins. However, the characteristics of the protein structure (Figs. 5, 6) may offer useful clues to its physiological function in cold acclimation. cDNA sequence analysis revealed that Picg5 proteins were novel ones with a few unusual properties common to a growing number of families of cold-regulated proteins (Thomashow 1999): high hydrophilicity, biased amino acid composition, and a repeat amino acid sequence motif capable of forming amphipathic α-helices (Figs. 5A, 6B, C).

Many plants with freezing tolerance have evolved various adaptive mechanisms against dehydration due to freezing. Cold-induced proteins are possibly associated with the adjustment of plant metabolisms, or have cryoprotective properties during cold acclimation (Artus et al. 1996; Thomashow 1999). The charged amino acids account for 42.2% of total amino acid residues in Picg5a (Fig. 4), similar to a cold-induced wheat protein WCOR410, which is proposed to scavenge ions and to counteract the harmful effects of increased ionic concentration during desiccation (Danyluk et al. 1998). Group II LEAs/dehydrins with the feature of conserved K-segments are hypothesized to stabilize macromolecules against freezing damage during cold acclimation (Close 1997). A peach (*Prunus persica*) dehydrin protein PC60 has been demonstrated to have both cryoprotective and antifreeze activity (Wisniewski et al. 1999). Like dehydrin K-segments, the core sequence of QKA repeats of Picg5a/b is predicted to form amphipathic α-helices (Fig. 6). Membrane damage is mainly caused by the transition of lipids from lamellar phase into hexagonal II phase (Uemura et al. 1995). The α-amphipathic helices in *Arabidopsis* cold-induced protein COR15a have been shown to inhibit the formation of the hexagonal II phase (Artus et al. 1996). Given the intracellular localization due to the absence of targeting signals and the accumulation during cold acclimation, Picg5 may have a similar role to other amphipathic proteins in stabilizing membranes or partially denatured proteins via hydrophobic interactions.

A typical dehydrin cDNA has been cloned from white spruce, which confirms that K-segments of dehydrins are highly conserved between conifers and angiosperms (Richard et al. 2000). However, only low similarity is found between QKA segments of Picg5 and K-segments of dehydrins (Fig. 6B). The antibody against dehydrin K-segment (Close 1997) was not able to recognize Picg5 proteins in western blot analysis (data not shown). Furthermore, its special expression pattern is different from most dehydrins, suggesting that the physiological activity of Picg5 may be different from dehydrins under freezing stress.

The other possible function of Picg5 may be to act as a proteinaceous compatible solute similar to other proteins reported to reduce the toxic effects of cellular solutes at high concentrations and to alleviate the potential damage caused by dehydration (Baker et al. 1988). During dehydration from freezing the loss of water brings about crystallization of molecular components in plant cells leading to the damage of cellular structures. In white spruce, freeze-desiccation is the most likely cause of observed overwintering injury (Krasowski et al. 1996). The randomly coiled motifs in some LEA proteins, such as D11 and D113, are proposed to bind water for the "solvation" of cytosolic structures by acting as compatible solutes (Baker et al. 1988). Picg5 are highly hydrophilic proteins. About half of their amino acid residues corresponding to the peaks of six hydrophilic domains are predicted to form random coils (Fig. 6). Therefore, cold-induced proteins such as Picg5 with coil structure properties could help maintain the minimum water required for the survival of plant cells under dehydration conditions associated with freezing (McCubbin and Kay 1985; Dure 1993).

DNA duplication or deletion in a gene sequence has also been reported in allelic genes containing repeat sequences such as dehydrin (Choi et al. 1999) and rehydrin (Velten and Oliver 2001). Polymorphism of *Picg5* nucleotide sequences and Picg5 proteins may represent very similar alleles of the same gene. This is further supported by the genomic DNA hybridization that suggests only one or two copies of Picg5 genes in the white spruce genome (Fig. 7). The insertion or deletion of DNA fragments followed by site mutations, as has occurred in the Picg5 gene may result in a protein with new biochemical properties and functions.

A significant seed lot effect on overwintering injury was found in white spruce, indicating a potential for genetic selection of families and clones resistant to the freezing injury from dehydration (Krasowski et al. 1996). The observed polymorphism of Picg5 proteins and *Picg5* cDNAs may provide a starting point for identification of molecular markers for breeding of freeze tolerance.

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