Antifreeze Evaluation of Two Dehydrin Proteins from *Pseudotsuga menziesii* and *Larix principis-rupprechtii* Mayr

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Abstract: Dehydrins exist widely in plants and play an important role in abiotic stress resistance. Two low-temperature-induced dehydrin-like genes, *PmCAP* and *LpCAP*, from the pine species *Pseudotsuga menziesii* and *Larix principis-rupprechtii* Mayr were cloned and found to contain 576 bp and 687 bp, encoding 191 and 228 amino acids, respectively. Both genes were individually assembled into prokaryotic expression vectors and transferred into *E. coli* cells. When transgenic stains were cultured at −5 °C, the lethal time 50% (LT50) was 50 h and 54 h for *PmCAP* and *LpCAP*, respectively, compared with 32 h for the control. When cultured at −20 °C, the LT50 was 38 h, 41 h, and 25 h for *PmCAP*, *LpCAP*, and the control. Thermal hysteresis (TH) testing of *PmCAP* and *LpCAP* proteins revealed TH values of 0.27 °C and 0.72 °C, respectively, relative to 0.05 °C for the BSA control. These results indicate that the two pine dehydrin proteins have antifreeze characteristics and that their antifreeze levels were well in relation to the environmental conditions of pine growth (*Larix principis-rupprechtii* Mayr mostly grows in cold and high-altitude zones, while *Pseudotsuga menziesii* grows in temperate and low-altitude zones). *LpCAP*, especially, could be a better gene resource for the molecular breeding of plant cold resistance.

Keywords: dehydrin; prokaryotic expression; thermal hysteresis; pine trees; antifreeze

1. Introduction

Dehydrin is a group II late embryogenesis abundant (LEA) protein with at least one Lys-rich K-segment 15 amino acids in length (EKKGMDKIKEKLPG) that can form an amphipathic α-helix [1,2]. The number of K-segments in the peptide is thought to be important for dehydrins’ hydrophily [3]. With the amphipathic α-helix structure, dehydrins can interact with other proteins or the dehydration surface of a biofilm to enhance and stabilize the confirmation and prevent protein dehydration. Dehydrin can also act as a molecular chaperone to inhibit protein polymerization and enable correct protein folding, thus maintaining the original cell shape and preventing cell death from excessive crumbling from stress [4]. Dehydrin is thought to play a role in defense against various abiotic stresses [5], such as heat [6], drought [7], flood [8], salinity [9], and low temperature [10].

Cuevas-Velazquez reported that dehydrin’s amphipathic α-helix surface can change the surface charge distribution in low temperatures and bind with ice crystals to prevent its recrystallization [11]. The growth shape of the ice crystals then changes to a hexagonal configuration, replacing the original large ice crystal structure, and decreases the ice crystal’s effect on plants’ physiological and metabolic activity. A citrus dehydrin gene was introduced into tobacco plants [12], which then exhibited lower relative electrolytic leakage than wild plants when exposed to −4 °C for 3 h as well as earlier germination and better seedling growth when exposed to 15 °C. Several other dehydrin genes, such as...
DHN24 from potato [13], WCOR410 from wheat [14], and RcDhn5 from rhododendron [15], have also been annotated with a dehydrin function in chilling/freezing resistance. Some prokaryotic expression assays provide further evidence for the role of dehydrin. For example, the insect AFP gene increases Escherichia coli survival rates when exposed to freeze stress [16], and AnAFP from Ammopiptanthus nanus keeps E. coli cells alive longer at 0 °C relative to the control [17].

Pinaceae plants are well known for their strong tolerance and acclimation to chilling, freezing, shading, and barren. This study aimed to identify putative cold-induced dehydrin genes from two pine species, *Pseudotsuga menziesii* (mainly growing in very cold areas) and *Larix principis-rupprechtii* Mayr (mainly growing in temperate regions) and evaluate their antifreeze properties via a prokaryotic expression system. The assays increased our understanding of the survival mechanism of pine trees in low temperatures and contribute to the antifreeze gene resources of plant molecular breeding programs.

2. Materials and Methods

2.1. Plant Materials and Bacterial Strains

One-year-old plants of *Pseudotsuga menziesii* and *Larix principis-rupprechtii* Mayr were grown in pots with sandy soil in a growth chamber at regular temperature (25 °C). Plants were irrigated daily and fertilized weekly with half-strength Hoagland’s nutrient solution [18]. Temperature treatments were conducted at 25 °C, 5 °C, 0 °C, −5 °C, and −20 °C, respectively. The leaves were collected for physiological indexes test at −20 °C treatment for 0, 4, and 8 days. The total RNA was extracted using TRIzol purification system (Invitrogen, Waltham, MA, USA) from leaves at 25 °C, 5 °C, 0 °C, and −5 °C treatment for 24 h, which were further reversely transcribed to cDNA using M-MLV reverse transcriptase and random primers (Promega, Madison, WI, USA) and used for dehydrin gene cloning and expression test.

2.2. Measurements of Physiological Indexes

Relative electrolyte leakage (REL): 1 g needles were collected and cut into 0.5 cm fragments, immersed in 40 mL deionized water, and shaken overnight. The electrical leakage of the solution was measured as R1. It was then boiled for 15 min and shaken overnight. The electrical leakage of the solution was measured again as R2. The relative electrolyte leakage was calculated using the formula of R1/R2 × 100%.

Malondialdehyde (MDA) content: 0.5 g leaf tissue was homogenized in 1 mL of 10% trichloroacetic acid (TCA). After centrifugation, the supernatant was collected to mix with 0.6% thiobarbituric acid (TBA) solution. The mixture was boiled for 10 min, immediately cooled on ice, and centrifuged. Absorbance of the supernatant at 450 nm, 532 nm, and 600 nm was measured, respectively. The MDA content was calculated using the formula of (6.45(A532 − A600) − 0.56A450) × total extract volume/total fresh sample weight.

The experiments were repeated three times. The gained data were assessed by Statistical Analysis System (SAS) (SAS, North Carolina, NC, USA) PROC ANOVA; the test model was Student Newman–Keuls Test (SNK) (\(p = 0.01\), \(p = 0.05\)).

2.3. Cold-Induced Dehydrin Gene Cloning from Pine Trees

Based on the dehydrin genes identified in the genome sequence library of *Pinus taeda* (axid: 3352), a pair of degenerate primers (Pin-F/Pin-R) were designed (Pin-F: 5′-GGTGTG-GATAAARTSAAAG-3′; Pin-R: 5′-CCCCGCTTCTGATTTGGATT-3′) and used for cold-induced dehydrin gene cloning from the pine trees. The PCR conditions were 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and extension at 72 °C for 10 min. Ubiquitin was as the reference gene (Pin UBQ-F, 5′-GCTTTATTCTCCGTCCA T3; Pin UBQ-R, 5′-TTTTCCTCGACTCCGCCC3). The PCR fragments were collected and cloned to pGEM-T easy vector (Promega, Madison, WI, USA).
2.4. Prokaryotic Expression Vector Construction

A pair of primers were designed with EoRI or XhoI restriction enzyme site inside for the dehydrin gene in *Pseudotsuga menziesii* (P-EcoRI, 5’-CCG-GAATTCATGGCTAAAAATGTAGCATCCG-3’, EcoRⅠrecognition site underlined; P-XhoI, 5’-CCGCTCGAGCTACTTTGAATCAGATTGAGTAGTC-3’, XhoⅠrecognition site underlined) and for the dehydrin gene in *Larix principis-rupprechtii* (L-EcoRI, 5’-CCG-GAATTCATGGCTGAAAATGTATCATC-3’, EcoRⅠrecognition site underlined; L-XhoI, 5’-CCGCTCGAGTTCCTTATTGCCTCCGGG-3’, XhoⅠrecognition site underlined). The full-length gene fragment was amplified with the cloned plasmid, recovered, digested with double enzymes, and ligated to the prokaryotic expression vector pET-28b (digested with same enzymes). The recombinant was transformed into the prokaryotic expression strain *E. coli* BL21 (DE3) by electroporation method. The putative transformed strain was determined by the specific PCR test.

2.5. Antifreeze Analysis of the Transgenic Strains

Strain growth curve was assessed as follows: The activated bacteria solution was inoculated in a fresh antibiotic-free LB liquid medium and cultivated for 3–4 h until OD600 in 0.6–0.8. IPTG was then added to a final concentration of 0.5 mM for 3–4 h for protein induction. The culturing bacteria solution was diluted 106-folds, sub-packed into 18 Eppendorf tubes, and shaken at −5 °C or −20 °C individually. One tube was taken out every other day, in which 100 µL bacteria solution was coated on LB agar at 37 °C overnight. The growth bacterial clones were counted, and the survival rate was calculated with the following formula: the plaques number in freezing treatment/the plaques number in control (before freezing treatment) [19]. The lethal time 50% (LT50) time for each strain was determined when the growth plaques number in freezing treatment was half of the control (no freezing treatment). The assay was repeated three times. The gained data were processed for ANOVA analysis as above.

2.6. Extraction and Purification of Target Proteins

The activated bacteria strains were inoculated in 1000 mL fresh LB medium and cultivated until OD600 in 0.6–0.8. IPTG was added to a final concentration of 0.5 mM and kept for 3–4 h. The bacterium was collected by centrifuge, resuspended in lysis buffer (20 mM Tris–HCl, 500 mM NaCl, 10% glycerinum), and treated with ultrasonic crusher for 15 min. Through the nickel chelate affinities chromatography, the crude extract was obtained, filtrated with Amicon Ultra 10 kD centrifuge filter unit (0.22 µm) (Millipore; Billerica, MA, USA), and performed chromatography with molecular sieve buffer (20 mM Tris pH 8.0, 100 mM NaCl, 40 mM imidazole) at 0.4 mL/min flow rate. The peak proteins were collected and detected through 15% polyacrylamide gel electrophoresis on sodium dodecyl sulfate (SDS-PAGE).

2.7. Thermal Hysteresis (TH) Assays of Target Proteins

The purified proteins were desalted with double-distilled water (ddH2O), freeze-dried into powders, and then dissolved in ddH2O. The protein concentration was determined using Bradford protein assay [20]. The thermal hysteresis activity (THA) of 10 mM target proteins was measured using the differential scanning calorimetry (DSC Q2000, TA, New Castle, DE, USA) with bovine serum albumin (BSA) as the control that does not have the antifreeze capability [21]. The process was as follows: 10 µL protein sample was tested from 25 °C to −30 °C (in which proteins were in a completely frozen solid phase), then heated to 10 °C (in which proteins were in a completely molten liquid phase), and cooled to −30 °C at 1 °C/min rate. An empty aluminum pan was the control. The melting enthalpy (∆Hm), melting point (Tm), retention temperature (Tr), recrystallization temperature (To), and recrystallization enthalpy (∆Hf) were recorded. The gained data were analyzed with
TA Universal Analysis 2000 software (TA Instruments, New Castle, DE, USA). Ice content (Φ) and THA were calculated with the formula: \( Φ = (1 - ΔH_m/ΔH_f) \times 100\% \), THA = \( T_h - T_o \).

3. Results

3.1. Antifreeze Evaluation of Pseudotsuga Menziesii and Larix Principis-rupprechtii Mayr

One-year-old plants of *Pseudotsuga menziesii* and *Larix principis-rupprechtii* Mayr were treated at \(-20^\circ C\) for 0, 4, and 8 days. The leaves were collected for REL and MDA content tests. The results showed that both pine tree species displayed a little decline as the treatment time went on. *Larix principis-rupprechtii* Mayr was more resistant to freezing than *Pseudotsuga menziesii* since the REL value increased by 240.35% and 279.05% relative to the control (0 days) when measured on days 4 and 8, respectively, whereas those of *Pseudotsuga menziesii* increased by 294.76% and 330.05%. The MDA content of *Larix principis-rupprechtii* Mayr increased by 201.39% and 388.31% relative to the control on days 4 and 8, respectively, while those of *Larix principis-rupprechtii* Mayr increased by 238.59% and 440.27% (Figure 1).
Figure 1. Assessment of freezing resistance of *Pseudotsuga menziesii* and *Larix principis-rupprechtii* at −20 °C for 0, 4, and 8 days via the relative electrolyte leakage (REL) (A) and malondialdehyde (MDA) content (B) evaluation. * and ** represent the significant difference between the treatment (4 d or 8 d) and the control (0 d) at *p* = 0.05 or *p* = 0.01.

3.2. Cloning and Sequence Analysis of Cold-Induced Dehydrin Genes

Degenerate primers (Pin-F/Pin-R) were used to amplify a fragment from cDNA samples collected from the pine tree species after −5 °C treatment; these were named *PmCAP* and *LpCAP*, respectively. Both genes were expressed in a cold-induced pattern, 7.22 folds and 2.44 folds at −5 °C, respectively, relative to the expression at 25 °C (Figure 2).

Figure 2. Assessment of dehydrin gene expression in *Pseudotsuga menziesii* and *Larix principis-rupprechtii* Mayr at 25 °C, 5 °C, 0 °C, and −5 °C with the degenerate primes (Pin-F/Pin-R). *Ubiqutin* gene was used as an internal control.
PmCAP was 576 bp and possibly encoded a 191 amino acid peptide with a theoretical molecular weight of 20.61 kD and isoelectric point of 5.39 (Figure 3). LpCAP was 687 bp, encoding a 228 amino acid peptide with a theoretical molecular weight of 24.52 kD and isoelectric point of 5.39. PmCAP and LpCAP had hydrophobic amino acids in a proportion of 71.2% and 70.2%, respectively. They are hydrophilic proteins since their hydrophobicity values were mostly negative (Figure 4). The gene sequences were deposited in GenBank (accession number: PmCAP OQ280978; LpCAP OQ280994).

**Figure 3.** Coding sequences in nucleotides and amino acids of PmCAP and LpCAP genes. ATG is the start codon. * is the stop codon.
Figure 4. The hydrophobicity analysis of PmCAP (black) and LpCAP (blue) proteins. X-axis displayed the amino acid order of the proteins. Y-axis displayed the hydrophobicity of each amino acid, where the positive value means hydrophobic and the negative value means hydrophilic.

PmCAP and LpCAP have typical characteristics of dehydrin proteins and individually contain four and five QKA fragments, respectively (Figure 5). Both genes are homologous with the *Pinus taeda* dehydrin gene APFE01090036.1, with which they have a 78% and 74% amino acid identity, respectively, whereas there was a 41% identity between the PmCAP and LpCAP peptides (78% for nucleotide sequence). The secondary structures of both PmCAP and LpCAP consisted of an alpha-helix, beta-turn, and random coil. The proportion of each structural component differed between species and was 35.60%, 5.24%, and 57.07% in PmCAP and 46.49%, 4.39%, and 49.12% in LpCAP, respectively, with a 2.09% extension strand plus in PmCAP (Figure 5).

Figure 5. Sequence alignment of PmCAP and LpCAP (A) and their secondary structures (B).
3.3. Prokaryotic Expression of PmCAP and LpCAP Genes

Both CAP genes were individually assembled into the prokaryotic expression vector pET-28b and then transformed to the BL21 E. coli strain (Figure 6A). The dehydrin proteins were expressed by IPTG induction, isolated, and separated by SDS-PAGE (Figure 6B). The protein products were consistent with the predicted CAP protein size, indicating that the CAP genes are properly translated in E. coli.

![Prokaryotic expression assessment](image)

**Figure 6.** Prokaryotic expression assessment. (A) Vector construction of CAP gene; (B) SDS-PAGE electrophoresis results of crude protein extract. M, Marker; BL21, wild E. coli strain; BT, transgenic BL21 strain with empty vector pET28b; BP1/BP2: transgenic BL21 strain with target gene PmCAP/LpCAP; +/−, with or without IPTG induced. Black arrows indicate the candidate proteins.

3.4. Antifreeze Evaluation of Transgenic E. coli Containing PmCAP and LpCAP

The strains were coated and cultured in solid medium at −5 °C for 2 days. The transgenic strains had high survival rates of 52.23% for PmCAP and 57.45% for LpCAP relative to 30.26% for the control (empty vector strain) (Figure 7A). The median lethal time (LT50) for the control was approximately 32 h, while that for the transgenic strains was 50 h for PmCAP and 52 h for LpCAP. Similar assays were conducted at −20 °C for 2 days. The survival rate of the control was 4.41% but that of the transgenic strains was 37.03% for PmCAP and 43.24% for LpCAP (Figure 7B). The LT50 for PmCAP, LpCAP, and the control was 38 h, 40 h, and 25 h, respectively. Both CAP genes showed antifreeze characteristics, with LpCAP being a little more effective than PmCAP.
Figure 7. Growth curve of strains at −5 °C (A) and −20 °C (B). Survival rate = number of colony at −5 °C (or −20 °C)/number of colony at 25 °C. Means of three independent experiments are shown in charts (p < 0.05); BL21, wild E. coli strain; BT, transgenic BL21 strain with empty vector pET28b; BP/BP2: transgenic BL21 strain with target gene PmCAP/LpCAP; +, IPTG added; −, no IPTG.

3.5. Purification and Thermal Hysteresis (TH) Test of PmCAP and LpCAP Proteins

PmCAP and LpCAP proteins were purified from the transgenic strains by isolation, ultrasonication, affinity chromatography (Figure 8), and molecular sieve. The final products had a target protein purity of over 95% (Figure 8C).

Figure 8. Purification of the LpCAP protein. (A) The preliminary purification results of protein (M, Marker; 1, total protein solution; 2, supernatant after ultrasonication; 3, preliminary purified protein). (B) His-affinity chromatography, (C), SDS-PAGE electrophoresis (1 and 2 are two repeats).

The purified proteins were used for differential scanning calorimeter (DSC) measurement. As the temperature increased, the crystal content in the protein and BSA control samples gradually decreased. In contrast, the TH curves for PmCAP and LpCAP protein solutions before recrystallization were similar to the control. When the solutions were frozen again, the DSC curve immediately appeared as a smooth exothermic peak. No delay was observed at the crystalizing temperature for the control (Figure 9A), but a sharp directional change was observed in the DSC curve of both CAP proteins (Figure 9B,C), which slowed the ice crystallization process and showed the TH reaction of the antifreeze proteins.
Additionally, both CAP proteins and the BSA control were displayed differently at both the freezing point (T0) and the retention temperature (Th) (Table 1). The thermal hysteresis ability (THA) varied greatly, with that of LpCAP at 2.67 times PmCAP and 14.40 times the BSA control. This indicated that CAP proteins can inhibit ice crystallization growth and help protect the cell from freezing injuries. LpCAP had a better protective ability than PmCAP.

Table 1. DSC characters of BSA, PmCAP, and LpCAP (each 10 mg·mL$^{-1}$).

<table>
<thead>
<tr>
<th>DSC Characters</th>
<th>BSA</th>
<th>PmCAP</th>
<th>LpCAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention temperature (Th/°C)</td>
<td>−0.2</td>
<td>−0.42</td>
<td>−0.80</td>
</tr>
<tr>
<td>Recrystallization temperature (T0/°C)</td>
<td>−0.25</td>
<td>−0.69</td>
<td>−1.52</td>
</tr>
<tr>
<td>Ice enthalpy (ΔHf/J·g$^{-1}$)</td>
<td>215.42</td>
<td>132.96</td>
<td>131.75</td>
</tr>
<tr>
<td>Total heat enthalpy (ΔHm/J·g$^{-1}$)</td>
<td>222.5</td>
<td>137.28</td>
<td>137.28</td>
</tr>
<tr>
<td>Ice nucleation (Nuclei/%) *</td>
<td>3.18</td>
<td>3.15</td>
<td>4.03</td>
</tr>
<tr>
<td>Thermal hysteresis ability (THA/°C)</td>
<td>0.05</td>
<td>0.27</td>
<td>0.72</td>
</tr>
</tbody>
</table>

* Nuclei (%) = [1 − (ΔHf)/ΔHm] × 100%; THA = T0 − Th.

4. Discussion

Dehydrin genes are widely present in plant genomes. Their encoded proteins can alleviate the impact of a freeze injury and provide individuals with antifreeze capabilities. For example, four dehydrin genes were cloned from spruce plants. A bacterial culture assay showed that the transgenic strains had a survival rate of between 33% and 53% when treated at −20 °C for 2 days, compared to 5% for the control [22]. The dehydrin gene *PicW* was cloned from *Picea wilsonii* Mast and introduced into tobacco plants. The overexpression of the gene enabled the plants to grow well at −5 °C for 3 h relative to wild plants. The relative electrical conductivity and MDA content decreased by 51% and 34% compared to wild plants, respectively, while the proline content and soluble content both increased by 35%. Also, the dehydrin genes *LTI29* and *LTI30* were transformed into *Arabidopsis thaliana*. The survival rate of the transgenic plants after 8 h of treatment at −10 °C was 94% compared to only 50% in the wild-type [23]. Comparably, our study evaluated two dehydrin genes from two pine species. The transgenic *E. coli* strains expressing these genes showed higher survival rates of 37.03% and 43.24% at −20 °C for 2 days in comparison to 4.4% for the control [24]. We therefore concluded that dehydrin performance is important for overwintering plants. Next, we will test their antifreeze role in plants.

TH activity is an important parameter to assess the antifreeze abilities of proteins by inhibiting ice crystallization. To date, dehydrin proteins from several plant species have had their THA values estimated, including *Secale cereale* L. (0.33) [25], *Ammopiptanthus nanus* (0.46) [26], *Prunus persica* (0.06) [27], *Picea wilsonii* Mast (0.77, PicW1; 0.78, PicW2) [22], *Pseudotsuga menziesii* (0.27, PmCAP), and *Larix principis-rupprechtii* Mayr (0.72, LpCAP) in this study. The THA for some non-dehydrin proteins has also been reported, including polygalacturonase inhibitor protein from *Hippophae rhamnoides* (0.19) [28], Class I chitinase from *Chimonanthus praecox* L. (0.52) [29], chitinase from *Picea abies* (2.19) [30].
and *Picea pungens* (2.02) [31], and agglutinin from *Ammopiptanthus mongolicus* (0.9) [32]. We concluded that proteins from different plant species have different antifreeze capabilities. Surveying more species would be useful for building an effective gene resource for molecular breeding programs on cold tolerance.

The various THA values of the proteins, meaning different antifreeze capabilities, were related to their protein composition and structure. Several studies have shown that the α-helix in the dehydrin protein might play an important role in its antifreeze activity. Chakrabarty found that the AFP protein affects the a- and c-axis growth rates of ice crystals. A highly helical synthetic AFP analog, which has its helical component increased by 21%, affects ice crystal growth rates at 7– to 8-fold lower concentrations than the native AFP. Possibly, the high degree of helix significantly enhances the affinity of AFP to ice crystals [33]. Fairley (2002) synthesized an AFP from *Myoxocephalus scorpius* with two extra residues appended to the N-terminus that resulted in a α-helical loop with the first four residues more flexible than the remainder of the sequence. The altered protein modified ice growth and confirmed that the N-terminus of the wild-type polypeptide is functionally important [34]. Pinus is an overwintering species with strong antifreeze abilities. We cloned and compared the antifreeze capability of two dehydrin genes from two Pinus species. LpCAP had a higher α-helix proportion of 46.49% compared to that of 35.60% in PmCAP. Transgenic assays and THA testing showed that LpCAP was more resistant to low temperatures than PmCAP. These results correspond well with the α-helix data of previous studies. The antifreeze difference seen between PmCAP and LpCAP corresponds to the species' native habitat, *Larix principis-rupprechtii* Mayr mostly grows in dry, cold, and high-altitude zones (1500 m and above), while *Pseudotsuga menziesii* grows in rainy, temperate, and low-altitude zones (800 m and above). Long-term environmental adaptation is likely to have led to the corresponding changes in dehydrin proteins. The relative mechanisms of the antifreeze differences between the genes are noteworthy. We will investigate their key amino acids and protein structure next.

5. Conclusions

The dehydrin-like genes from two pine species were revealed to have different antifreeze levels via prokaryotic expression assays and thermal hysteresis tests of proteins, which might result from the long-term environmental adaptation of the Pinus plants. They can be important gene resources for plant antifreeze breeding.

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Data Availability Statement: Publicly available datasets were analyzed in this study. This data can be found in GenBank [accession number: PmCAP OQ280978; LpCAP OQ280994].

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Conflicts of Interest: The authors declare no conflict of interest.

References


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