PicW2 from *Picea wilsonii*: preparation, purification, crystallization and X-ray diffraction analysis

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Low temperature is a major limiting factor for plant growth and development. Dehydrin proteins are generally induced in response to low-temperature stress. In previous research, a full-length dehydrin gene, *PicW2*, was isolated from *Picea wilsonii* and its expression was associated with hardiness to cold. In order to gain insight into the mechanism of low-temperature tolerance by studying its three-dimensional crystal structure, prokaryotically expressed PicW2 dehydrin protein was purified using chitosan-affinity chromatography and gel filtration, and crystallized using the vapour-diffusion method. The crystal grew in a condition consisting of 0.1 M HEPES pH 8.0, 25%(w/v) PEG 3350 using 4 mg ml⁻¹ protein solution at 289 K. X-ray diffraction data were collected from a crystal at 100 K to 2.82 Å resolution. The crystal belonged to space group C121, with unit-cell parameters $a = 121.55$, $b = 33.26$, $c = 73.39$ Å, $\alpha = \gamma = 90.00$, $\beta = 109.01^\circ$. The asymmetric unit contained one molecule of the protein, with a corresponding Matthews coefficient of 2.87 Å³ Da⁻¹ and a solvent content of 57.20%. Owing to a lack of structures of homologous dehydrin proteins, molecular-replacement trials failed. Data collection for selenium derivatization of PicW2 and crystal structure determination is currently in progress.

1. Introduction

Low temperature is a major limiting factor for plant growth and development (Boyer, 1982). Plants have evolved a variety of molecular responses to survive low temperatures. Many genes have been found to be related to low-temperature stress and include those encoding transcription factors, protein kinases, radical-scavenging enzymes, water channels, late embryogenesis abundant (LEA) proteins *etc.* (Seki *et al.*, 2002). Despite the identification of numerous low-temperature-induced genes in a wide range of plant species, it is still unclear how they contribute to the tolerance of low temperature; in particular, the contribution of LEA proteins remains far from being completely understood.

Dehydrin (DHN) proteins (LEA DII family) are generally induced in response to a wide array of environmental stimuli (Campbell & Close, 1997). DHN proteins have been the most commonly observed among the proteins that accumulate in response to low temperature (Close, 1997). DHN proteins are present in many organisms, and the first DHN sequence to be published was that of the RAB21 protein from rice (Mundy & Chua, 1988). Many DHN proteins have subsequently been identified that contribute to low-temperature tolerance, such as PCA60 in peach (*Prunus persica*; Wisniewski *et al.*, 2010),

CuCOR19 in orange (*Citrus unshiu*; Hara *et al.*, 2001) and ZmDHN2b in maize (*Zea mays* L. zheng 958; Xing *et al.*, 2011). Three highly conserved domains, the K, Y and S segments, were commonly characterized in DHN proteins (Close, 1997). The K segment is usually located in the C-terminus and is composed of a highly conserved 15-amino-acid motif (EKKGIMDKIKEKLP; Close, 1996). The Y segment (T/VDEYGNP), an N-terminal consensus sequence, shares partial homology with the nucleotide-binding sites of chaperones in plants and bacteria (Close, 1997). The S segment includes a phosphorylatable tract of serine residues (Vilardell *et al.*, 1990) which is necessary for binding to nuclear localization signal peptides (Godoy *et al.*, 1994). According to the composition of the three conserved domains, DHN proteins can be divided into five categories (YnSK, Kn, KnS, SKn and YnK; Allagulova *et al.*, 2003). The amino-acid sequences of the K segments demonstrate the possibility of the formation of a class A amphipathic α -helix (Baker *et al.*, 1988). This might interact with exposed hydrophobic patches of partially denatured proteins and thus resemble chaperones. Porat *et al.* (2004) identified two genes, *csDHN* (*Citrus sinensis* DHN) and *cpDHN* (*C. paradisi* DHN), which encode the typical plant angiosperm-type K-segment consensus sequence, and their expression of mRNA was increased by exposure to low temperature levels. Thus, DHN proteins may carry out a protective function at low temperatures and their mechanism may differ from those of other proteins. This difference in mechanism may be related to the structure of the K segment in DHN proteins.

Wilson spruce (*Picea wilsonii*), a high-mountain species that occurs at elevations between 1400 and 3000 m, is an important timber tree in China. It is commonly planted as an amenity tree in northern China and Russia, and grows well in low-temperature regions. Therefore, the identification of low-temperature-induced proteins or genes in Wilson spruce would meet both basic and economic research interests and provide an understanding of the mechanisms for the tolerance of low temperature.

In previous research, the full-length DHN gene *PicW2* was isolated from *P. wilsonii* and its expression was associated with cold stress (Liu *et al.*, 2014). The sequence of the *PicW2* gene was analyzed and the result showed that several nucleic acid substitutions and deletions could change the secondary structure of the *PicW2* protein. The sequence variations could also affect the capability to tolerate low temperatures (Zhao *et al.*, 2017). In order to provide an understanding of the mechanisms for the tolerance of low temperature, in this research we focus on the expression, purification and crystallization of the *PicW2* protein.

2. Materials and methods

2.1. Macromolecule production

Genomic DNA was extracted from the needles of *P. wilsonii* (Zou *et al.*, 2006). The *PicW2* gene was cloned by Liu *et al.* (2014). The expression vector pTwin1-*PicW2* was produced as

Table 1

Macromolecule-production information.

Source organism	<i>P. wilsonii</i>
DNA source	Accession No. AY220128
Forward primer	5'-CCGGAATTCATGGCTGAAATGTATC G-3'
Reverse primer	5'-CGCGGATCCTCATTCCTTGTCTCTGGG C-3'
Cloning vector	pEASY-Blunt
Expression vector	pTwin1
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MAENVSRRLGEGKEEESGRDQGVMRPA TTNTQSQSAYPNAAQKATLVVDKIKEML PGSQTDQAQSDHSPNEAEATAYPDA AQKATLLDKVDLLPESQTETKAQTVH SNPNEAETQTAYPNAAQKATLVVDKIKD ILPGSQTETEAQTAHSPNQETETETET QTGYPNAAQKATLVVDKVEGMFTGGQTE TDTETAQSNPNQKAGLVVDKIKENIPRA QNKE

described by Zhao *et al.* (2017). Briefly, the gene for *PicW2* was amplified using PCR techniques and was then ligated into the BamHI or EcoRI restriction sites of the pTwin1 vector, resulting in the recombinant vector pTwin1-*PicW2*. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) competent cells. Macromolecule-production information is summarized in Table 1.

Positive transformants were grown in 5 ml LB medium containing 100 $\mu\text{g ml}^{-1}$ ampicillin overnight at 310 K and were subcultured into 1000 ml LB medium pH 7.0 containing 100 $\mu\text{g ml}^{-1}$ ampicillin. Protein expression was induced by the addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 8–10 h at 289 K when the OD₆₀₀ reached 0.6–0.8. The cells were harvested and the bacterial pellet was resuspended in lysis buffer (20 mM HEPES pH 8.0, 500 mM NaCl) with 1 mM phenylmethanesulfonyl fluoride (PMSF) and the cells were then lysed by sonication. The clear supernatant,

Figure 1

Purification of *PicW2* on a gel-filtration column and SDS-PAGE analysis. (a) 12% SDS-PAGE stained with Coomassie Brilliant Blue. Lane M, protein marker (labelled in kDa); lane 1, *PicW2* corresponding to the peak on the gel-filtration profile in (b). (b) Purification profile of *PicW2*, which eluted as a symmetrical peak from the Superdex G200 SEC column.

Table 2
Crystallization.

Method	Vapour diffusion
Plate type	48-well Double Sample Crystallization Plate (screening); 16-well Crystallization Plate (final)
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	4
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 100 mM NaCl
Composition of reservoir solution	0.1 mM HEPES pH 7.5, 25%(w/v) PEG 3350 (screening); 0.1 mM HEPES pH 8.0, 25%(w/v) PEG 3350 (final)
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir	80 µl (screening); 400 µl (final)

after centrifugation at 13 000 rev min⁻¹ for 30 min at 277 K, was applied directly onto a 2 ml Chitin Fast Flow column (Biolabs) equilibrated with 20 mM HEPES pH 8.0, 500 mM NaCl, 0.1 mM EDTA. The contaminant proteins were washed out with wash buffer (20 mM HEPES pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100). The target protein was eluted with elution buffer (20 mM HEPES pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 50 mM DTT) and shaken for 48 h at 277 K. The eluted protein was collected, concentrated to 2 ml and applied onto a Superdex G200 (GE Healthcare) size-exclusion chromatography (SEC) column equilibrated with buffer consisting of 20 mM Tris-HCl pH 8.0, 100 mM NaCl. The target peak was assessed by SDS-PAGE (Fig. 1).

2.2. Crystallization

The PicW2 protein was concentrated to 4 mg ml⁻¹ for crystallization. Crystallization screening of PicW2 was initially performed at 289 K with commercially available kits from Hampton Research (Crystal Screen, Crystal Screen 2, Index, PEGRx1, PEGRx2, PEG/Ion and PEG/Ion 2) using the sitting-drop vapour-diffusion method in a 48-well Double Sample Crystallization Plate. The drop size was 2 µl, consisting of 1 µl protein solution mixed with an equal amount of reservoir solution, and was equilibrated against 80 µl reservoir

Figure 2
Crystal of PicW2 obtained from a condition consisting of 0.1 M HEPES pH 8.0, 25%(w/v) PEG 3350.**Table 3**
Data collection and processing.

Values for the outer shell are given in parentheses.

Diffraction source	3W1A, BSRF
Wavelength (Å)	0.9792
Temperature (K)	100
Detector	MAR165 CCD
Crystal-to-detector distance (mm)	160
Rotation range per image (°)	1
Total rotation range (°)	200
Exposure time per image (s)	45
Space group	C121
<i>a</i> , <i>b</i> , <i>c</i> (Å)	121.55, 33.26, 73.39
α , β , γ (°)	90.00, 109.01, 90.00
Mosaicity (°)	0.606
Resolution range (Å)	50.00–2.82 (2.87–2.82)
Total No. of reflections	22414
No. of unique reflections	6472
Completeness (%)	92.1 (98.3)
Multiplicity	3.5 (3.0)
$\langle I/\sigma(I) \rangle$	11.43 (2.49)
<i>R</i> _{int} (%)	26.15 (51.82)
Overall <i>B</i> factor from Wilson plot (Å ²)	41

solution. After the initial crystallization condition (Index No. 44) had been obtained, optimization of the crystallization condition was performed by fine-tuning the pH in the range 6.5–8.5 and the PEG 3350 concentration in the range 15–35% using the hanging-drop vapour-diffusion method in a 16-well Crystallization Plate at 289 K. A suitable single crystal for data collection (Fig. 2) was finally obtained after 90 d in a condition consisting of 0.1 mM HEPES pH 8.0, 25%(w/v) PEG 3350. Crystallization information is provided in Table 2.

2.3. Data collection and processing

The harvested crystals were soaked for a few seconds in reservoir solution containing 15%(v/v) glycerol and were quickly mounted on the goniometer in a nitrogen stream at 100 K. Data collection was performed with a step size of 1.0° using a MAR165 CCD detector on beamline 3W1A at the Beijing Synchrotron Radiation Facility synchrotron-radiation source, Institute of High Energy Physics, Chinese Academy of Sciences. The data were indexed, integrated and scaled with *HKL-2000* (Otwinowski & Minor, 1997) and processed using the *CCP4* suite (Winn *et al.*, 2011).

3. Results and discussion

In previous research, the sequence of PicW2 was analyzed and showed that several amino-acid substitutions or deletions could change the secondary structure of the PicW2 protein, which led to further changes in its tolerance of low temperature (Zhao *et al.*, 2017). To explore the relationship between the three-dimensional crystal structure and low-temperature tolerance mechanism of PicW2, prokaryotically expressed PicW2 protein was purified using chitosan-affinity chromatography and gel filtration, and crystallized using the vapour-diffusion method. PicW2 was found to have a molecular weight of 24.4 kDa (Fig. 1*a*) and was purified to homogeneity,

Figure 3
An image of the PicW2 diffraction pattern to a resolution of 2.82 Å.

eluting as a symmetrical peak from the Superdex G200 column (Fig. 1*b*) at 17.22 ml elution buffer.

We obtained initial crystallization in Index condition No. 44 consisting of 0.1 M HEPES pH 7.5, 25%(w/v) PEG 3350. Subsequent optimization was performed in 16-well hanging-drop plates and a suitable single crystal for data collection was finally obtained after 90 d in a condition consisting of 0.1 M HEPES pH 8.0, 25%(w/v) PEG 3350 (Fig. 2). A total of 200 images with an oscillation angle of 1° each were collected with an exposure time of 45 s per frame (Fig. 3). The crystal belonged to space group C121, with unit-cell parameters $a = 121.55$, $b = 33.26$, $c = 73.39$ Å, $\alpha = \gamma = 90.00$, $\beta = 109.01^\circ$. The asymmetric unit was estimated to contain one molecule of PicW2, with a corresponding Matthews coefficient of $2.87 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 57.20%. The data-collection statistics are summarized in Table 3.

To acquire further structural information on the PicW2 protein, we performed molecular replacement (MR) using *MOLREP* in the *CCP4* suite (Winn *et al.*, 2011). We used the amino-acid sequence of PicW2 to search the Protein Data Bank (PDB) and no results were found matching our sequence. We then input the amino-acid sequence of PicW2 into the *SWISS-MODEL* server. Among the homologous structures obtained by the *SWISS-MODEL* server, we selected the eight top-ranked crystal structures exhibiting

sequence identities of 13.64% to PicW2 as potential search models. However, all MR trials with those models failed. We used *Iterative Threading ASSEMBLY Refinement (I-TASSER)* to search for structural templates, but none were found by the *I-TASSER* server. Data collection from a selenium derivative of PicW2 and crystal structure determination are currently in progress.

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