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Crystallization and preliminary crystallographic analysis of a calcineurin B-like protein 1 (CBL1) mutant from *Ammopiptanthus mongolicus*

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Calcineurin B-like protein 1 (CBL1) is a calcium sensor in plants. It transmits the calcium signal through the downstream protein CBL-interacting protein kinase (CIPK). CBL1 and CIPK play crucial roles in the response to environmental stresses such as low K^+, osmotic shock, high salt, cold and drought. Recombinant CBL1 from *Ammopiptanthus mongolicus* (*Am*CBL1) was overexpressed, purified and crystallized. However, the crystal did not diffract well. A mutant prepared using the surface-entropy method and crystallized using the hanging-drop method at 298 K with PEG 2000 MME as a precipitant diffracted to 2.90 Å resolution. The crystal belonged to space group $P2_12_12$, with unit-cell parameters a = 99.87, b = 114.42, c = 63.80 Å, $\alpha = \beta = \gamma = 90.00^\circ$ and three molecules per asymmetric unit.

1. Introduction

Unlike animals, plants cannot escape adverse environmental stresses. Active reaction to the stress is the only way that they can survive and grow. A series of sophisticated mechanisms have evolved in plants to deal with biotic and abiotic stress. Messengers are often used to transduce the stress signal from the extracellular environment to the interior and to elicit responses at the cellular and whole plant level. Calcium is one of the common messengers. Its cellular concentration fluctuates when the surrounding environment changes (Bush, 1995; Trewavas & Malho, 1998; Rudd & Franklin-Tong, 2001). Calcium signalling involves sensor proteins that decode temporal and spatial changes in cellular Ca^{2+} concentration. Three families of calcium-signalling proteins have been identified in higher plants. The first family consists of calmodulin (CaM) and CaM-related proteins (Zielinski, 1998; Snedden & Fromm, 2001). The second major class consists of Ca^{2+}-dependent protein kinases (CDPKs; Roberts & Harmon, 1992; Harmon *et al.*, 2000). CDPKs that contain a CaM-like Ca^{2+}-binding domain and a protein kinase domain are unique owing to the existence of a Ca^{2+} sensor and effector in a single protein. Calcineurin B-like protein (CBL) and CBL-interacting protein kinase (CIPK) form a novel calcium signal-decoding system (Albrecht *et al.*, 2003; Cheong *et al.*, 2003; Pandey *et al.*, 2004; Luan, 2008; Batistic & Kudla, 2009). CBL, which is similar to the calcineurin B subunit and neuronal calcium sensors (NCS) from animals, changes its conformation on binding to calcium and activates self-inhibited CBL-interacting protein kinase (CIPK; Liu & Zhu, 1998; Kudla *et al.*, 1999; Shi *et al.*, 1999; Halfter *et al.*, 2000). CIPK is a type of Ser/Thr kinase that phosphates downstream effector proteins such as ion channels or transcription regulators that depress or activate the expression of certain stress-related genes (Qiu *et al.*, 2002; Quintero *et al.*, 2002; Li *et al.*, 2006; Xu *et al.*, 2006; Lee *et al.*, 2007).

The CBLs identified in *Arabidopsis thaliana* (*At*CBL1–10) and rice (*Os*CBL1–10) by genome annotation show a high degree of similarity: 33–93% in *A. thaliana* and 44–94% in rice (Kolukisaoglu *et al.*, 2004). All CBLs possess a classical or nonclassical EF-hand Ca^{2+}-binding motif (Lewit-Bentley & Rety, 2000). *At*CBL2 is mainly responsible for light stimuli and strongly interacts with *At*CIPK14 (Chikano *et al.*, 2001; Nozawa *et al.*, 2001). In concert with PKS5

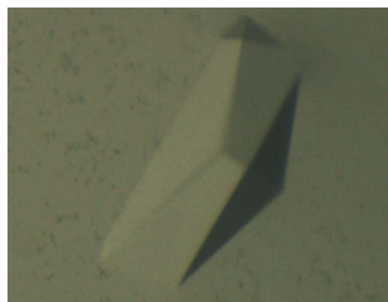

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Table 1
Primers for site-directed mutation.

Primer name	Primer sequence
*Am*CBLFM66	5′-AGATATTTGCTGCTGCCCTATTTTTGAAAATTG-3′
*Am*CBLRM66	5′-CAAAAATAGGGCAGCAGCAAATATCTTTGCAAAT-3′
*Am*CBLFM82	5′-GACTCCTGCCGCCGCAACATCAAATAG-3′
*Am*CBLRM82	5′-TTTGATGTTGCGGCGGCAGGAGTCATTGAT-3′
*Am*CBLFM141	5′-AGCCAACGCCATTGCAGACTCACAAAG-3′
*Am*CBLRM141	5′-GAGTCTGCAATGGCGTTGGCTGAT-3′

(CIPK11), *At*CBL2 negatively regulates the activity of AHA2, a plasma-membrane H^+-ATPase (Fuglsang *et al.*, 2007). Salt stress strongly induces the expression of *At*CBL4 or salt overly sensitive 3 (SOS3; Liu & Zhu, 1998). *At*CBL4/SOS3 acts with *At*CIPK24 (SOS2) to activate the plasma-membrane Na^+ transporter SOS1 through phosphorylation (Qiu *et al.*, 2002; Zhu, 2002). *At*CBL4/SOS3 functions mainly in plant roots, but its homologue SOS3-like Ca^{2+}-binding protein 8 (SCABP8)/calcineurin B-like protein 10 (CBL10) functions in salt tolerance in shoot and leaf tissue (Quan *et al.*, 2007; Kim *et al.*, 2007). *At*CBL5 is expressed in green tissues. It may function as a positive regulator in high-salt, drought or osmotic stress signalling pathways (Cheong *et al.*, 2010). Like *At*CBL5, *At*CBL1 also functions as a positive regulator for drought and salt but functions as a negative regulator in cold response (Cheong *et al.*, 2003). Although *At*CBL1 has a similar amino-acid sequence to *At*CBL9, it is not involved in the ABA signalling pathway in which *At*CBL9 plays a dominant role (Albrecht *et al.*, 2003; Pandey *et al.*, 2004). In recent years, a low-K^+ response signalling pathway in which *At*CBL1, *At*CBL9 and their target protein CIPK23 participate has been discovered. Low-K^+ status can trigger elevated production of H_2O_2 in plant cells. Accumulation of H_2O_2 changes the concentration of Ca^{2+} in the cytoplasm. *At*CBL1 and *At*CBL9 saturated with Ca^{2+} bind the NAF domain/FISL motif of *At*CIPK23 and then activate this protein kinase. Activated *At*CIPK23 phosphorylates the K^+ transporter AKT1 that pumps K^+ from the extracellular environment to the cell, which finally leads to low-K^+ tolerance and regulates the stomatal opening of guard cells (Li *et al.*, 2006; Xu *et al.*, 2006; Lee *et al.*, 2007; Cheong *et al.*, 2007; Luan *et al.*, 2009).

To date, structures of *At*CBL2 (Nagae *et al.*, 2003) and its complex with the regulatory domain of *At*CIPK14 (Akaboshi *et al.*, 2008) and of *At*CBL4 (Sánchez-Barrena *et al.*, 2005) and its complex with the C-terminus of *At*CIPK24 (Sánchez-Barrena *et al.*, 2007) have been determined. These structures provide clues to understanding the relationship between calcium concentration and the calcium-binding capability of certain CBLs, *i.e.* the specialities of different CBLs in decoding the various calcium signals from different environmental stresses. Although CBL1 plays a distinct role in the CBL family, there is no structure available. In this paper, we report the expression, purification and crystallization of the CBL1 from *Ammopiptanthus mongolicus* (*Am*CBL1), which has 82% identity to *At*CBL1. The native crystal of *Am*CBL1 did not diffract well, but a mutant form prepared using the low surface-entropy method diffracted to 2.90 Å resolution. Its preliminary X-ray analysis is reported here.

2. Materials and methods

2.1. Molecular cloning

*Am*CBL1 from *A. mongolicus* (GenBank accession No. AY902246.2) with a deletion of the N-terminal 17 amino acids was amplified by polymerase chain reaction using the forward primer 5′-GAAATT**CATATG**CCAGTAATTCTTGCATCAC-3′ and the reverse primer 5′-CCG**CTCGAG**AGCAGCAACTTCATCCAC-3′ (restriction sites are shown in bold). The PCR product was digested with the restriction enzymes *Nde*I and *Xho*I and inserted into pET-22b(+) (Novagen), resulting in the vector pET-22b-*Am*CBL1Δ17 with a 6×His tag added to the C-terminus to facilitate the purification procedure. Six mutants (*Am*CBL1Δ17-1 with K66A, K67A and E68A mutations; *Am*CBL1Δ17-2 with K82A, K83A and K84A mutations; *Am*CBL1Δ17-3 with E141A and K143A mutations; *Am*CBL1Δ17-12 with K66A, K67A, E68A, K82A, K83A and K84A mutations; *Am*CBL1Δ17-23 with K82A, K83A, K84A, E141A and K143A mutations; and *Am*CBL1Δ17-123 with K66A, K67A, E68A, K82A, K83A, K84A, E141A and K143A mutations) were constructed with pET-22b-*Am*CBL1Δ17 as the template using the gene splicing by overlap extension (gene SOEing) method (Horton *et al.*, 1990). The primer pairs used for the mutation of Lys66, Lys67 and Glu68 were named *Am*CBLFM66 and *Am*CBLRM66, those for the mutation of Lys82, Lys83 and Lys84 were named *Am*CBLFM82 and *Am*CBLRM82 and those for the mutation of Glu141 and Lys143 were named *Am*CBLFM141 and *Am*CBLRM141. The primer pairs used for the mutations are shown in Table 1. Constructs were verified by sequencing. Plasmids were transformed into *Escherichia coli* strain BL21 (DE3) for expression. The expression, purification and crystallization procedures used for the wild-type and mutant proteins were the same.

2.2. Protein expression and purification

10 ml aliquots of an overnight culture were subcultured into 1 l fresh LB medium containing ampicillin (50 µg ml^{-1}). When the OD_{600} reached 0.8 at 310 K, 0.25 ml 0.8 *M* IPTG was added to 1 l of culture. The protein was expressed overnight at 289 K. The bacteria were then harvested by centrifugation (30 min, 4000 rev min^{-1}). The bacterial pellet was resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 1 m*M* $CaCl_2$, 20 m*M* imidazole) and ultrasonicated on ice for 10 min. After centrifugation (30 min, 16 000 rev min^{-1}, 277 K), the clear supernatant was applied onto 5 ml Ni Sepharose Fast Flow (GE Healthcare) equilibrated with buffer *A*. Contaminant proteins were washed away using buffer *A*. The target protein was eluted with buffer *B* containing 20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl, 1 m*M* $CaCl_2$, 250 m*M* imidazole. The collected protein was concentrated to 2 ml and then applied onto a Superdex G200 size-exclusion chromatography (SEC) column (GE Healthcare) equilibrated with 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 1 m*M* $CaCl_2$,

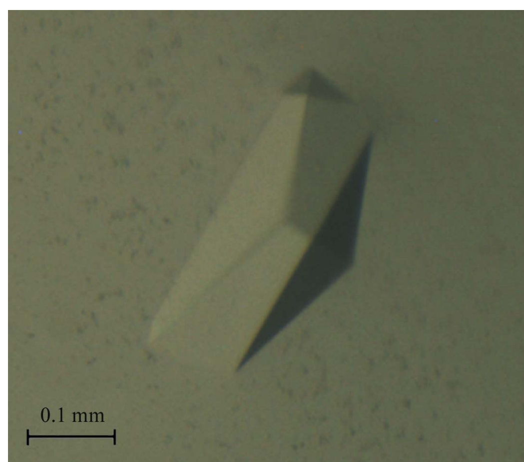

Figure 1
Typical crystal of *Am*CBL1Δ17-123.

1 mM dithiothreitol (DTT). The target peak was concentrated to 24 mg ml^{-1} for crystal growth.

2.3. Crystallization

Crystallization conditions were screened at 298 K by the vapour-diffusion method using commercial screening kits (Crystal Screen, Crystal Screen 2 and Index Screen; Hampton Research, USA) by mixing 1 µl protein solution and 1 µl reservoir solution and equilibrating against 400 µl reservoir solution in the well. After several days, flaky crystals of *Am*CBL1Δ17-123 appeared using Index Screen condition No. 78 (0.1 M bis-tris pH 5.5, 0.2 M ammonium acetate, 25% PEG 3350). The crystal quality was improved when 7% PEG 2000 MME was substituted for 25% PEG 3350 (Fig. 1).

2.4. Data collection and processing

The harvested crystals of *Am*CBL1Δ17-123 were soaked in dehydration solution consisting of 0.1 M bis-tris pH 5.5, 0.2 M ammonium acetate, 7% PEG 2000 MME, 0.7 M Li_2SO_4 for 5 min and then dipped into reservoir solution containing 15%(w/v) glycerol for several seconds and quickly mounted on the goniometer in a nitrogen stream at 100 K. Data were collected using a MAR 345 CCD detector on beamline BL17U1 at the SSRF synchrotron-radiation source, Shanghai, People's Republic of China. The data collected were indexed, integrated and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). Data-collection statistics are listed in Table 2.

3. Results and discussion

The whole length of *Am*CBL1 was not expressed in *E. coli* because of its N-myristoylation motif. Therefore, this flexible disordered region (amino acids 1–17) was deleted, resulting in N-terminally truncated *Am*CBL1Δ17 and finally leading to overexpression in *E. coli*. SDS–PAGE analysis of purified *Am*CBL1Δ17-123 with a 6×His tag at the C-terminus (theoretical molecular mass of 23.1 kDa) showed that its purity was above 90% and that its molecular mass was approximately 23 kDa (Fig. 2). *Am*CBL1Δ17 was crystallized with good morphology using a condition consisting of 0.2 M calcium acetate, 0.1 M sodium cacodylate pH 6.5, 18% PEG 8000 with 1% Triton X-305 as an additive. However, the crystal did not diffract at all. It is believed that protein crystallization predominately depends on the entropic effect. The entropic cost of releasing ordered water from the sites of crystal contacts is countered by ordering of the protein molecules, *i.e.* the loss of freedom of side chains on the protein surface. Mutational protein crystal-contact engineering was designed to reduce the surface entropy and has proved to be a successful way of enhancing the crystallizability of proteins and improving crystal quality (Derewenda, 2004). Three clusters of residues (cluster 1, residues 66, 67 and 68; cluster 2, residues 82, 83 and 84; cluster 3, residues 141 and 143) with high entropy and low evolutionary conservation were selected for mutational crystal-contact engineering tests. All of the residues in these clusters were changed to alanine, which has a low conformational entropy. Six mutants (*Am*CBL1Δ17-1, *Am*CBL1Δ17-2, *Am*CBL1Δ17-3, *Am*CBL1Δ17-12, *Am*CBL1Δ17-13, *Am*CBL1Δ17-23 and *Am*CBL1Δ17-123) were constructed, purified and screened for crystallization according to the protocol described above. These mutants produced a variety of crystals, which were tested for

Table 2
X-ray diffraction data-collection and processing statistics.

Values in parentheses are for the outermost shell.

Space group	$P2_12_12$
Wavelength (Å)	0.97928
Resolution range (Å)	30.00–2.90 (3.00–2.90)
Unit-cell parameters (Å)	a = 99.87, b = 114.42, c = 63.80
No. of observed reflections	233073
No. of unique reflections	16883
$\langle I/\sigma(I)\rangle$	54.6 (4.4)
Completeness (%)	99.5 (95.9)
R_{merge}† (%)	17.7 (43.5)
Matthews coefficient (Å^3 Da^{-1})	2.7
Solvent content (%)	53.6
No. of molecules in asymmetric unit	3

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the mean value of $I(hkl)$ for all i measurements.

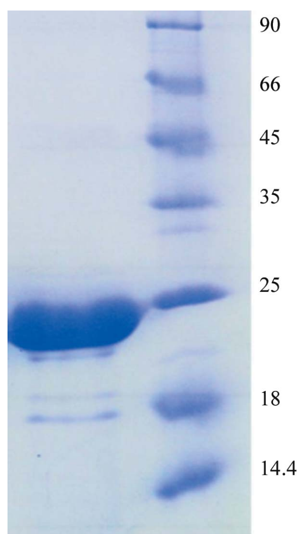

Figure 2
15% SDS–PAGE analysis of *Am*CBL1Δ17-123. Left lane, *Am*CBL1Δ17-123 after size-exclusion chromatography; right lane, molecular-mass markers (labelled in kDa).

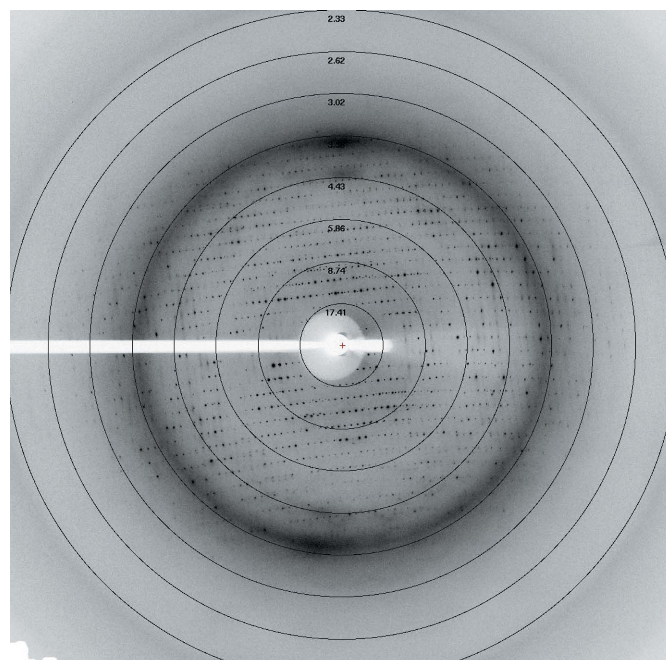

Figure 3
Diffraction pattern of *Am*CBL1Δ17-123 to 2.90 Å resolution.

diffraction. The shape and diffraction of six mutant crystals was compared and showed that the number of mutated residues may affect the growth of crystals. A larger number of mutated residues on the protein surface lowers the surface entropy and results in better crystal growth and diffraction to higher resolution. Finally, a mutant combining all three clusters (*Am*CBLΔ17-123) produced single crystals with good diffraction. Owing to the high solvent content of this crystal, dehydration with 0.7 *M* Li_2SO_4 was performed. Raw data were collected to 2.90 Å resolution (Fig. 3). The crystal belonged to space group $P2_12_12$, with unit-cell parameters a = 99.87, b = 114.42, c = 63.80 Å. The asymmetric unit is estimated to contain three molecules of *Am*CBL1Δ17-123, with a corresponding Matthews coefficient of 2.7 $Å^3$ Da^{-1} and a solvent content of 53.6%. Structure determination is currently under way.

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