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Crystallization and preliminary X-ray diffraction analysis of the flax cytokinin oxidase LuCKX1.1

Li Wan,[a] Simon J. Williams,[a] Xiaoxiao Zhang,[a] Daniel J. Ericsson,[a] Markus Koeck,[b] Peter N. Dodds,[b] Jeffrey G. Ellis[b] and Bostjan Kobe[a]*

[a]School of Chemistry and Molecular Biosciences, Institute for Molecular Bioscience (Division of Chemistry and Structural Biology) and Australian Infectious Diseases Research Centre, University of Queensland, Brisbane, Queensland 4072, Australia, and [b]Plant Industry, CSIRO, Canberra, ACT 2601, Australia

Correspondence e-mail: b.kobe@uq.edu.au

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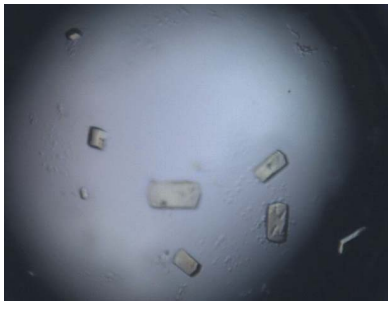

The plant hormones cytokinins play a central role in regulating cell division and developmental events. Cytokinin oxidase regulates the levels of these plant hormones by catalyzing their irreversible oxidation, which contributes to the regulation of various morpho-physiological processes controlled by cytokinins. In this study, the crystallization and preliminary X-ray diffraction analysis of the flax cytokinin oxidase LuCKX1.1 are reported. Plate-like crystals of LuCKX1.1 were obtained using PEG 3350 as a precipitant and diffracted X-rays to 1.78 Å resolution. The protein crystals have the symmetry of space group *C*2 and are most likely to contain two molecules per asymmetric unit.

1. Introduction

The plant hormones cytokinins are adenine-derived compounds substituted with an isoprenoid or aromatic side chain at the N6 position. Cytokinins play a central role in regulating cell division and specific developmental events, such as shoot and root branching, leaf development and chloroplast ripening (Ha *et al.*, 2012; Hwang *et al.*, 2012). Cytokinins have also been shown to play a role in plant–microbe interactions and disease resistance and susceptibility (Choi *et al.*, 2010; Pertry *et al.*, 2009, 2010).

Cytokinin oxidases or cytokinin dehydrogenases (CKXs) are enzymes that contain flavin adenine dinucleotide (FAD) as a cofactor and are responsible for most cytokinin catabolism within the plant. They cleave the N6 side chain of the hormone (Brownlee *et al.*, 1975; Whitty & Hall, 1974). Changes in the activity of cytokinin oxidases will cause the cytokinin concentration in tissues to change, which contributes to the regulation of various morpho-physiological processes controlled by cytokinins. Cytokinin oxidases are encoded by multigenic families with varying numbers of genes. For instance, there are seven genes coding for cytokinin oxidases (AtCKX1–AtCKX7) in the *Arabidopsis* genome, 13 genes coding for cytokinin oxidases (ZmCKX1–ZmCKX13) in the maize genome and at least 11 genes of cytokinin oxidases (OsCKX1–OsCKX11) in the rice genome (Schmülling *et al.*, 2003; Vyroubalová *et al.*, 2009; Ashikari *et al.*, 2005). The cytokinin oxidase genes have been characterized best in maize plants. ZmCKX genes exhibit different tissue specificity and transcription profiles, while their protein products differ in terms of enzymatic activity, substrate specificity, subcellular localization and glycosylation (Smehilová *et al.*, 2009; Vyroubalová *et al.*, 2009). The differences found in these enzymes suggest that they play a role in the coordination of various functions of cytokinins. Of the ZmCKX proteins, ZmCKX1 is the best characterized. The crystal structure of ZmCKX1 has been determined in several states, including in complex with reaction products and a slowly reacting substrate (Malito *et al.*, 2004). Substrate binding does not cause any obvious conformational change in the structure. The only other available structure is that of *Arabidopsis* cytokinin oxidase AtCKX7, which has been determined only in its FAD-bound state (Bae *et al.*, 2008). ZmCKX1 and AtCKX7 share a sequence identity of only 39.4%, but exhibit very similar two-domain topology structure with a root-mean-square distance (r.m.s.d.) of 1.8 Å for 474 equivalent C^{α} atoms (*DaliLite*; Holm & Park, 2000). FAD is covalently linked to a conserved histidine residue. Residues identified in ZmCKX1 as important for the binding of FAD and substrates are conserved in AtCKX7.

Table 1
Crystallographic data-collection and processing statistics.

Values in parentheses are for the outer shell.

Diffraction source	Australian Synchrotron MX2
Wavelength (Å)	0.953691
Temperature (K)	100
Detector	ADSC Quantum 315r CCD
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	0.5
Total rotation range (°)	360
Space group	*C*2
Unit-cell parameters (Å, °)	a = 201.54, b = 58.93, c = 91.23, α = 90.00, β = 111.55, γ = 90.00
Mosaicity (°)	0.16
Resolution range (Å)	85.08–1.78 (1.88–1.78)
Total No. of reflections	716846
No. of unique reflections	95714
Completeness (%)	99.8 (98.4)
Multiplicity	7.5 (7.3)
Mean $I/\sigma(I)$	17.8 (2.8)
R_{meas}	0.100 (0.796)
R_{merge}	0.093 (0.740)

LuCKX1.1 is one of about 16 cytokinin oxidases from flax (*Linum usitatissimum*) and has been found to interact with the flax rust (*Melampsora lini*) effector AvrL567 (Koeck *et al.*, unpublished work). To understand the possible role of this protein as a potential virulence target and to complement our work on the structure and function of flax rust effector proteins and the flax resistance proteins (Gunčar *et al.*, 2007; Wang *et al.*, 2007; Ve, Williams, Stamp *et al.*, 2011; Ve, Williams, Valkov *et al.*, 2011; Bernoux *et al.*, 2011), we embarked on structural analysis of LuCKX1.1. Here, we report the crystallization of recombinant LuCKX1.1 and the preliminary X-ray diffraction analysis of the crystals.

2. Materials and methods

2.1. Cloning, protein expression and purification

Fragments encoding residues 3–525, 3–534, 44–525 and 44–534 were amplified by PCR from full-length LuCKX1.1 cDNA and inserted into the pMCSG7 vector using ligation-independent cloning (Stols *et al.*, 2002). The resulting constructs contained an N-terminal His_6 tag followed by a TEV (*Tobacco etch virus*) protease cleavage site. The integrity of the constructs was confirmed by sequencing. Solubility tests in *Escherichia coli* BL21 (DE3) cells demonstrated that only the construct consisting of residues 44–534 of LuCKX1.1 (termed simply LuCKX1.1 hereafter) was soluble. This construct lacked the N-terminal region of LuCKX1.1 and the corresponding part was found to be disordered in the AtCKX7 structure (Bae *et al.*, 2008). The protein was expressed in *E. coli* BL21 (DE3) cells using auto-induction medium (Studier, 2005) containing 100 µg ml^{-1} ampicillin for plasmid selection. Cells were grown by continuous shaking at 310 K until the $OD_{600\ nm}$ reached 0.6–0.8. The temperature was then decreased to 293 K and the cells were further grown at 293 K for 20 h before harvesting by centrifugation. The cells were resuspended in lysis buffer (50 m*M* HEPES pH 8.0, 500 m*M* NaCl, 1 m*M* DTT) and lysed using sonication. The cell debris and insoluble material were removed by centrifugation at 27 216*g* for 40 min at 277 K and the collected supernatant was loaded onto a 5 ml HisTrap column (GE Healthcare). To remove unbound proteins and contaminants, the column was washed with 20 column volumes of wash buffer (50 m*M* HEPES pH 8.0, 500 m*M* NaCl, 30 m*M* imidazole). Bound protein was eluted using a linear gradient of imidazole from 30 to 250 m*M* and the fractions containing the protein of interest were pooled and concentrated to 2 ml for TEV treatment at 277 K overnight to remove the His_6 tag. The 20 ml TEV cleavage buffer consisted of 50 m*M* Tris–HCl pH 8.0, 250 m*M* NaCl, 1 m*M* DTT, 0.5 m*M* EDTA, 1 mg TEV protease and 2 ml concentrated LuCKX1.1 protein sample. The cleaved LuCKX1.1 protein was re-applied onto the HisTrap column to remove the His_6 tag and other contaminants.

Flowthrough fractions containing cleaved LuCXK1.1 protein were collected, concentrated and applied onto a Superdex 200 HiLoad 26/60 gel-filtration column (GE Healthcare) pre-equilibrated with gel-filtration buffer consisting of 10 m*M* HEPES pH 7.5, 150 m*M* NaCl, 1 m*M* DTT. The peak fractions were pooled, concentrated to a final concentration of 5 mg ml^{-1} and stored in aliquots at 193 K for crystallization studies.

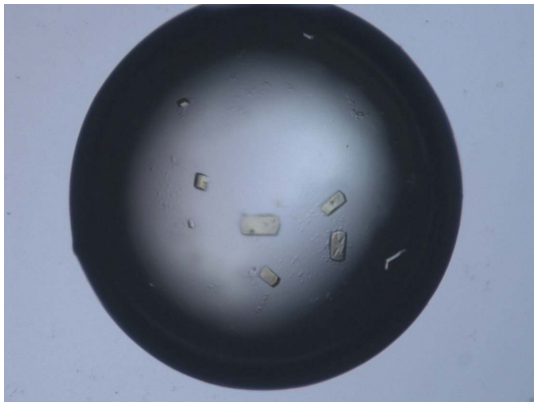

Figure 1
Plate-like crystals of LuCKX1.1 (100 × 40 × 8 µm) grown in 0.24 *M* tribasic ammonium citrate pH 8.0, 18%(*w*/*v*) PEG 3350.

2.2. Crystallization and X-ray data collection

The optimal protein concentration for crystallization was determined by the PCT screen (Hampton Research). Initial screening was conducted in 96-well plates (LabTech) at 293 K using the hanging-drop vapour-diffusion method. Eight commercial screens were utilized, including Index, PEG/Ion and PEGRx (Hampton Research), Morpheus, ProPlex, JCSG+ and Pact Premier (Molecular Dimensions) and Precipitant Synergy (Emerald BioSystems). Hanging drops consisting of 100 nl protein solution and 100 nl reservoir solution were set up using a Mosquito robot (TTP LabTech, UK) and were equilibrated against 100 µl reservoir solution. The drops were monitored and imaged using a Rock Imager system (Formulatrix, USA).

Optimization of hits from the initial screens was achieved by fine-tuning the protein concentration, the precipitant concentration, the pH and the size of the drop. Crystals of LuCKX1.1 were mounted with nylon loops and transferred to the mother liquor containing 25% glycerol prior to flash-cooling in liquid nitrogen. Data sets were collected from single crystals on the Australian Synchrotron MX2 beamline at a wavelength of 0.953691 Å using an ADSC Quantum 315r CCD detector. The crystal-to-detector distance was set to 250 mm, the oscillation range was 0.5° and 720 images were collected. Data were collected using the *Blu-Ice* software (McPhillips *et al.*, 2002), indexed and integrated using *XDS* (Kabsch, 2010) and scaled with *SCALA* within the *CCP*4 suite (Winn *et al.*, 2011).

3. Results and discussion

Four constructs of LuCKX1.1 with small N- and C-terminal deletions were made based on sequence alignment with AtCKX7 (59% sequence identity). The protein comprising residues 44–534 was produced in a soluble form in *E. coli*. After purification using immobilized metal-affinity chromatography and size-exclusion chromatography, the purity was estimated to be greater than 95% according to SDS–PAGE.

Initial crystallization screening was conducted at 293 K in 96-well plates using a protein concentration of 5 mg ml^{-1}. Small LuCKX1.1 crystals appeared after 1 d under several different conditions. One condition consisting of 0.2 *M* tribasic ammonium citrate pH 7.0, 20%(*w*/*v*) PEG 3350 (Index condition No. 88) was chosen for further optimization. After optimization, plate-like crystals were obtained in a solution consisting of 0.24 *M* tribasic ammonium citrate pH 8.0, 18%(*w*/*v*) PEG 3350 (Fig. 1). A data set was collected to 1.78 Å resolution from one of these crystals at the Australian Synchrotron. Data-collection statistics are given in Table 1. Structure determination is currently under way.

The structurally best-characterized cytokinin oxidase is ZmCKX1 from maize. The LuCKX1.1 and ZmCKX1 enzymes differ substantially (37% sequence identity) and the structures of the substrate and product complexes of LuCKX1.1 should uncover interesting differences in substrate binding and the catalytic mechanism between these enzymes. The structure should also shed light on its interaction with the flax rust effector protein AvrL567.

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