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Cloning, expression, purification, crystallization and preliminary crystallographic analysis of the kinase domain of AtMAP4Kalpha2 from *Arabidopsis thaliana*

Arabidopsis thaliana (At) MAP4Kalpha2, a member of the Ste20/PAK-like protein kinase family, is an essential component of the septum initiation network involved in cell division. To better understand the mode of action of AtMAP4Kalpha2, a structural biology approach has been pursued. In this study, the kinase domain of AtMAP4Kalpha2 was cloned, expressed, purified and crystallized. The crystals diffracted to 1.9 Å resolution and belonged to space group C222₁, with unit-cell parameters $a = 55.27$, $b = 82.93$, $c = 133.15$ Å.

1. Introduction

The members of the *Arabidopsis thaliana* Ste/PAK-like protein kinase family (AtMAP4Ks) are similar to yeast Ste20/PAK MAP4K, but their functions are still poorly characterized. The AtMAP4Ks terminology is derived from their ability to phosphorylate AtMAP3Ks (Champion, Picaud & Henry, 2004). The AtMAP4Ks can be classified into ten members and all of them have a conserved motif TFVGTPXWMAPEV (Jonak *et al.*, 2002). All of the AtMAP4Ks have an N-terminal catalytic domain and a diversified C-terminal domain, except for AtMAP4K3, in which the kinase domain is in the middle of the sequence.

AtMAP4Kalpha2 (GenBank ID 820753), systematic name proposed by Laszlo Bögre, was previously identified as a putative MAP4K (http://personal.rhul.ac.uk/ujba/110/mapk/map4k_genes.htm). It is highly expressed in seedlings, shoots, stems and roots (Moustafa *et al.*, 2008; Menges *et al.*, 2008). According to STRING prediction (Szklarczyk *et al.*, 2011), AtMAP4Kalpha2 possesses three functional partners: AtMAP4Kalpha1 (putative nuclear localization signal protein), LOV (putative disease resistance protein RPP8-like protein 1; Corchnoy *et al.*, 2003; Swartz *et al.*, 2001; Salomon *et al.*, 2000) and RPT2 (a signal transducer of the phototropic response and photo-induced movements; Motchoulski & Liscum, 1999).

AtMAP4Kalpha2 constitutes the central core of a septum initiation network (SIN)-related signalling pathway with AtSGP1 (*A. thaliana* spg1-like G protein) and AtMAP3Kepsilon1 (*A. thaliana* MAP kinase kinase kinase), which can directly control cell division in plants or assist other MAP kinases in controlling cell plate formation and cytoskeletal components in order to ensure cell plate expansion and cell division (Bedhomme *et al.*, 2008). In this signalling pathway, AtMAP3Kepsilon1, recruited by AtSGP1, is able to activate AtMAP4Kalpha2 (Champion, Jouannic *et al.*, 2004). However, the activation mechanism of AtMAP4Kalpha2 and how AtMAP4Kalpha2 regulates the downstream processes remain poorly reported. It has been reported that MST4, a human homologue of AtMAP4Kalpha2, is activated *via* its C-terminal domain, mediating homodimerization (Dan *et al.*, 2002; Qian *et al.*, 2001). MST4 and AtMAP4Kalpha2 share 63% sequence identity over the kinase domain (Fig. 1). Whether AtMAP4Kalpha2 is regulated in the same way is still unknown.

In the present study, we cloned, expressed, purified and crystallized the kinase domain of AtMAP4Kalpha2 (AtMAP4Kalpha2-KD), corresponding to amino acids 1–290. We obtained a diffraction data set which extended to 1.9 Å resolution.

MST4-KD (human)	MAHSPVQSGLPQMNLKADPEELFTKLEKIGKGSFGEVFKGIDNRTQKVVAIKIIDLEEA	60
MST4-KD (mouse)	MAHSPVQSGLPQMNLKADPEELFTKLEKIGKGSFGEVFKGIDNRTQKVVAIKIIDLEEA	60
AtMAP4Kalpha2-KD	-----MDDVAGLQEAAGARFSQTEILIGRGSFGDVYKAFDKDLNKEVAIKVIDLEES	51
MST4-KD (human)	EDEIEDIQQEITVLSQCDSPIYVTKYYSYKDKTKLWIIMEYLGGSALDLEPG-PLDET	119
MST4-KD (mouse)	EDEIEDIQQEITVLSQCDSPIYVTKYYSYKDKTKLWIIMEYLGGSALDLEPG-PLDEI	119
AtMAP4Kalpha2-KD	EDEIEDIQKEISVLSQCRCPIYTEYYSYHQTKLWIIMEYMAGGSVADLLQSNPLDET	111
MST4-KD (human)	QIATILREILKGLDYLHSEKKIHRDIKAANVLLSEHGEVKLADFGVAGQLTDTQIKRNTF	179
MST4-KD (mouse)	QIATILREILKGLDYLHSEKKIHRDIKAANVLLSEHGEVKLADFGVAGQLTDTQIKRNTF	179
AtMAP4Kalpha2-KD	SIACITRDLHAVEYLNHNEGKIHRDIKAANILLSENGDVKVADEFGVSAQLTRTISRRTF	171
MST4-KD (human)	VGTPFWMAPEVIKQS-AYDSKADIWSLGITAIELARGEPPHSELHPMKVLFILPKNNPPT	238
MST4-KD (mouse)	VGTPFWMAPEVIKQS-AYDSKADIWSLGITAIELARGEPPHSELHPMKVLFILPKNNPPT	238
AtMAP4Kalpha2-KD	VGTPFWMAPEVIQNSEGYNEKADIWSLGITVIEAMAKEPLADLHPMRVLFIIIPRETPPQ	231
MST4-KD (human)	LEGNYSKPLKEFVEACLNKEPSFRPTAKELLKHKFILRNAKKTSYLTTELIDR-----	290
MST4-KD (mouse)	LEGNYSKPLKEFVEACLNKEPSFRPTAKELLKHKFIIRNAKTSYLTTELIDR-----	290
AtMAP4Kalpha2-KD	LDEHFSRQVKEFVSLCLKKAPAEERPSAKELIKHRFIKNARKSPKLLERIRERPKYQVKE	290

Figure 1

Sequence alignment between AtMAP4Kalpha2-KD and homologous proteins from human and mouse.

Figure 2

AtMAP4Kalpha2-KD (lane 1) was identified by SDS-PAGE. Lane 2 contains molecular-mass markers (labelled in kDa).

2. Materials and methods

2.1. Molecular cloning

The coding sequence of AtMAP4Kalpha2-KD was amplified by polymerase chain reaction (PCR) from an *Arabidopsis* root cDNA library. The PCR product was digested by *Nco*I and *Not*I restriction enzymes and ligated into expression vector HT-pET-28a, which was modified from pET-28a (Novagen) and had an N-terminal 6×His tag followed by a TEV protease site. Positive clones were confirmed by DNA sequencing and the recombinant plasmids were transformed into *Escherichia coli* BL21(DE3) competent cells for expression.

2.2. Protein expression

E. coli BL21(DE3) competent cells were grown at 310 K in Terrific Broth (TB) medium to an OD₆₀₀ of 1.0 and isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to the culture medium to a final concentration of 0.5 mM to induce protein expression at 289 K. After 16 h, cells were harvested by centrifugation at 6000g for 10 min at 277 K. Cells were resuspended in lysis buffer (20 mM HEPES pH

7.5, 500 mM NaCl, 5% glycerol, 20 mM imidazole, 1 mM DTT) and then broken using a High Pressure Homogenizer (JNBIO). The cell suspension was sonicated and the supernatant was separated at 277 K by centrifugation (2000g, 30 min).

2.3. Purification

The supernatant was mixed with Ni Sepharose (GE Healthcare) pre-equilibrated with lysis buffer for 1 h; Ni Sepharose can capture the target 6×His-tagged fusion protein (HHHHHHHENLYFQ/**GAMG** +AtMAP4Kalpha2-KD, where the TEV sequence corresponds to the residues given in bold and **Q/G** is the TEV protease site) from the supernatant. The bound proteins were eluted using a linear gradient of 0–400 mM imidazole in lysis buffer. For further purification, the eluted protein was desalted to 20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT, 10 mM imidazole and incubated with TEV protease (purified in our laboratory) at 288 K for 2 h. The 6×His tag and TEV protease were then removed by Ni Sepharose again. The proteins were concentrated to less than 5 ml using a 10 kDa cutoff Amicon Ultra-15 (Millipore) and were further purified using a HiLoad 16/60 Superdex 75 column (GE Healthcare). The purity of the proteins was monitored by SDS-PAGE.

2.4. Crystallization

The AtMAP4Kalpha2-KD was concentrated to 5 mg ml⁻¹ in buffer consisting of 20 mM HEPES pH 7.5, 100 mM NaCl. Initial crystallization trials were carried out at 291 K using the sitting-drop vapour-diffusion method with Hampton Research screens (Crystal Screen, Crystal Screen 2, PEG/Ion, PEG/Ion 2, SaltRx, Index, Wizard I, Wizard II, Wizard III and Wizard IV). 2 µl sitting drops consisting of 1 µl protein solution and 1 µl reservoir solution were equilibrated against 100 µl reservoir solution. Prior to crystallization, 1 mM AMP-PNP and 1 mM MgCl₂ were added to the purified protein solution. Following extensive optimization trials, we refined the initial condition by varying the precipitant concentration within an established range [30–15% (w/v) of PEG 3350] and the buffer pH within an established range (HEPES pH 6.5–8.5). Crystals were grown in a solution consisting of 22% (w/v) PEG 3350, 0.1 M HEPES pH 8.0.

2.5. Data collection and processing

For X-ray data collection, native AtMAP4Kalpha2-KD crystals were soaked in a cryoprotection solution composed of 0.1 M HEPES

pH 8.0, 22% (w/v) PEG 3350, 16% glycerol and flash-cooled in liquid nitrogen. Diffraction data were collected on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF) in China. A total of 180 frames were collected with an oscillation step of 1° and 1 s exposure per frame. The crystal-to-detector distance was maintained at 250 nm. Diffraction data were processed and scaled using *HKL-2000* (Otwinowski & Minor, 2001).

3. Results and discussion

The *E. coli* BL2(DE3) strain containing the HT-pET-28a +AtMAP4Kalpha2-KD vector showed a high level of AtMAP4K-alpha2-KD expression in the soluble extract. The 6 $\times$ His-fused AtMAP4Kalpha2-KD was successfully digested with TEV protease. A crystallization-grade sample was obtained by two purification steps involving nickel-affinity chromatography and size-exclusion chromatography. The AtMAP4Kalpha2-KD used in crystallization

Table 1

Data-collection statistics for the AtMAP4Kalpha2-KD crystal.

Values in parentheses are for the outermost resolution shell.

Beamline	BL-17U1
Detector	MAR 225 CCD
Space group	C222 ₁
Unit-cell parameters (Å, °)	$a = 55.27$, $b = 82.93$, $c = 133.15$, $\alpha = \beta = \gamma = 90.0$
Wavelength (Å)	0.9793
Resolution range (Å)	50.0–1.90 (1.93–1.90)
Total reflections	176893
Unique reflections	24564
Completeness (%)	99.9 (100.0)
Multiplicity	7.2 (7.3)
$R_{\text{merge}}^\dagger$ (%)	0.071 (0.941)
$\langle I/\sigma(I) \rangle$	26.8(2.7)
Matthews coefficient (Å ³ Da ⁻¹)	2.18
Mosaicity (°)	0.260
Molecules per asymmetric unit	1
Solvent content (%)	44

$$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl).$$

(a)

(b)

Figure 3

(a) Single crystal of AtMAP4Kalpha2-KD. (b) X-ray diffraction image from an AtMAP4Kalpha2-KD crystal. The edge of the diffraction frame is at 1.9 Å resolution.

experiments was checked by SDS–PAGE analysis and had a purity of greater than 95% (Fig. 2).

Initial crystals of AtMAP4Kalpha2-KD appeared after 10 d using PEG 3350 as a precipitant. Various optimization procedures such as varying the precipitant concentration and the buffer pH were tried in order to obtain diffraction-quality crystals. Finally, crystals were obtained using the sitting-drop vapour-diffusion method with reservoir solution (Fig. 3a). X-ray diffraction data were collected from a single crystal to 1.9 Å resolution (Fig. 3b). A total of 176 893 measured reflections were merged into 24 564 unique reflections, giving an R_{merge} of 7.1% and a completeness of 99.9%. The reflections were indexed in the orthorhombic crystal system with unit-cell parameters $a = 55.27$, $b = 82.93$, $c = 133.15$ Å. An examination of the systematic absences indicated that the crystal belonged to space group C222₁. Calculation of the Matthews coefficient (Matthews, 1968) based on the molecular weight of 32.3 kDa resulted in a V_M of 2.18 Å³ Da⁻¹ and a solvent content of 44%, which corresponded to the presence of one molecule per asymmetric unit. The statistics of data processing are summarized in Table 1. We are currently attempting to solve the structure of AtMAP4Kalpha2-KD using molecular-replacement calculations with the atomic coordinates of MST4 (PDB entry 3ggf; Record *et al.*, 2010) as a search model. In parallel with crystallographic studies, kinase assays are being carried out in order to shed light on the kinase activity of the AtMAP4K-alpha2-KD.

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