Cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of the LRR domain of the LePRK1 receptor-like kinase from tomato

Anbi Xu^{a,b} and Laiqiang Huang^{b,c,*}

^aTsinghua–Peking Center for Life Sciences, Beijing 100084, People's Republic of China, ^bSchool of Life Sciences, Tsinghua University, Beijing 100084, People's Republic of China, and ^cThe Shenzhen Key Laboratory of Gene and Antibody Therapy, Division of Life and Health Sciences, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, People's Republic of China

Correspondence e-mail:
huanglq@tsinghua.edu.cn

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LePRK1 is a receptor-like kinase involved in successful fertilization in *Lycopersicon esculentum* (tomato). Importantly, the extracellular leucine-rich repeat (LRR) domain of LePRK1 mediates transmembrane signal transduction for pollen-tube growth and pollen germination. In this study, the N-terminal extracellular LRR domain of *L. esculentum*-derived LePRK1 was purified using an insect-cell secretion expression system and was crystallized by the vapour-diffusion method. The crystals diffracted X-rays to a resolution of 2.75 Å using synchrotron radiation. The crystals belonged to space group C2, with unit-cell parameters $a = 136.53$, $b = 56.01$, $c = 62.93$ Å, $\beta = 108.99^\circ$ and two molecules per asymmetric unit.

1. Introduction

Receptor-like kinases (RLKs) are a family of transmembrane receptors with an intracellular serine/threonine kinase domain and perform critical functions including individual growth, organ development and defence responses in both plants and animals. More than 600 RLK genes have been identified in the genome of *Arabidopsis* (Finn *et al.*, 2008; Letunic *et al.*, 2012; Sigrist *et al.*, 2010; Burge *et al.*, 2012).

RLKs consist of an extracellular domain, a single transmembrane segment and a cytoplasmic kinase domain. The extracellular domain is believed to sense and consequently transduce environmental signals, initiating subsequent activation of downstream signalling pathways *via* phosphorylation of the cytoplasmic kinase domain. Consistent with this idea, structural studies have confirmed the direct binding of ligands to the extracellular domains of their respective RLK receptors. RLKs are divided into seven subfamilies according to architectural differences within the extracellular domains. These include leucine-rich repeat receptor-like protein kinases (LRR-RLKs; Kobe & Deisenhofer, 1994), legume lectin receptor kinases (LecRLKs; Hervé *et al.*, 1996; Sharon & Lis, 1990), receptors with C-type motifs (Weis & Drickamer, 1996; Hawgood & Poulain, 2001), lysin motif receptor kinases (Bateman & Bycroft, 2000), chitinase-related receptor-like kinases (CHRK1s; Kim *et al.*, 2000), epidermal growth factors (EGFs), repeat receptor kinases (Rao *et al.*, 1995) and TNFR motif receptor kinases (Naismith & Sprang, 1998). Among these, LRR-RLKs constitute the largest subfamily of RLKs. To date, numerous LRR-RLKs that play crucial roles in the pollination of flowering plants have been identified (Tichtinsky *et al.*, 2003).

LePRK1 and LePRK2 are two LRR-RLKs that localize on the pollen-tube wall in tomato plants (*Lycopersicon esculentum*) and participate in the fertilization process (Muschietti *et al.*, 1998). Briefly, following germination and maturation, the mature pollen grains are released from the anther in a dehydrated state and land on the compatible stigma of the pistil. There, the pollen grains hydrate, causing the pollen tube to elongate and traverse the transmitting bundle of the style before finally arriving at an ovule to deliver the sperm. Successful pollination is necessary for the propagation of flowering plants and the continuation of genetic diversity. Therefore, the process of pollination is tightly regulated between pollen and pistil cells *in vivo*. Studies have shown that upregulation of LePRK1

in pollen will result in abnormal growth of the pollen tube. Detailed analysis has demonstrated that LePRK1 can initiate and integrate many different signalling pathways related to cytoskeleton remodelling and vesicle transportation to support the normal growth of pollen tubes (Tang *et al.*, 2011). LePRK2 functions as a cofactor with LePRK1 in the process of pollination.

LePRK1 is a 74 kDa protein with an extracellular domain (ECD), a single transmembrane segment and a cytoplasmic kinase domain (Fig. 1). The ECD is comprised of 251 amino acids (nearly 29 kDa), including a 20-amino-acid signal peptide (residues 1–20), a hydrophobic N-terminal sequence (LRRNT; 21–84), six LRRs in tandem (LRR; 85–227) and a hydrophobic C-terminal cap (LRRCT; 228–251). Studies have suggested that at various stages of pollen-tube growth LePRK1 interacts with different ligands to bring about distinct functional consequences. For example, an anther-specific protein, LAT52, interacts with LePRK1, which is essential for pollen hydration and pollen-tube growth (Tang *et al.*, 2002). *In vitro*, the LRR domain of LePRK1 (LePRK1-LRR) has been shown to interact with LeSHY (Tang *et al.*, 2004), a small pollen-specific protein (Guyon *et al.*, 2000) that is significantly increased in the initial stages of pollen-tube growth to moderate the signal transduction pathway. Additionally, LePRK1 can bind LeSTIG1, a female pistil protein. It currently remains unknown how LePRK1 is able to interact with multiple proteins for signalling. Structural characterization of LePRK1-LRR would provide clues to address this question. To this end, LePRK1-LRR was expressed in an insect-cell secretion expression system, purified and crystallized. The most similar homologue of known structure is FLS2 (PDB entry 4mn8; Sun *et al.*,

2013), with a sequence identity of 34% and a sequence similarity of 49%.

2. Materials and methods

2.1. Molecular cloning

Total RNA was purified from *L. esculentum* using the EZNA Total RNA Kit I (Omega). Reverse-transcriptase PCR was carried out with a One Step RT-PCR kit (Omega). The cloning primers for LePRK1-LRR (amino acids 1–251) were as follows: forward primer, LePRK1-*Bam*H1-F (5'-CGGGATCCATGTCGGTGGCTTATCGTTATAG-3'); reverse primer, LePRK1-*Sal*I-251-R (5'-GCGTCGACCAGAGCTA CTTTGGTTCAC-3'). The PCR product was digested with *Bam*HI and *Sal*I enzymes and cloned into pFastBac 1 vector (Invitrogen).

2.2. Protein expression and purification

Expression of LePRK1-LRR (residues 1–251) was carried out using the baculovirus insect-cell system. Briefly, LePRK1-LRR with an engineered C-terminal 6×His tag and a modified N-terminal haemolin secretory signal peptide was cloned into a pFastBac1 vector (Invitrogen; Fig. 2). The protein was expressed in *Spodoptera frugiperda* (Sf21) insect cells by incubation at 28°C. 1 l insect cells (2.5×10^6 cells ml⁻¹) was infected with 20 ml baculovirus and the protein was harvested from the medium 46 h after infection. The supernatant was bound to Ni-NTA (Novagen) and then eluted with buffer consisting of 25 mM Tris pH 8.0, 150 mM NaCl, 250 mM imidazole. The eluted protein was concentrated and further purified by size-

Figure 1

Secondary structure of the LRR-RLK LePRK1. The limits of the domains are indicated by the corresponding amino-acid numbers. Si-P, signal peptide. LRRNT, LRR N-terminal domain. LRR, leucine-rich repeat. LRRCT, LRR C-terminal domain. TM, transmembrane segment. K-D, cytoplasmic kinase domain.

Figure 2

The LePRK1-LRR-pFastBac1 expression construct. Haemolin peptide, the LePRK1-LRR sequence and the C-terminal 6×His tag are indicated. LePRK1 signal peptide (SI-P) is coloured red; the LRRNT domain and LRRCT domain are coloured green. The LRR domain was divided into six LRR units (LRR1–LRR6); conserved amino acids from the central LRR domain are shown in red on a yellow background. SI-P, signal peptide. LRRNT, LRR N-terminal domain. LRR, leucine-rich repeat. LRRCT, LRR C-terminal domain.

Figure 3

SDS-PAGE analysis of the LePRK1-LRR protein expressed in insect cells. The elution volume of the peak fraction is 14.74 ml. Lane P1, glycosylated LePRK1-LRR protein in the peak fraction from gel filtration (Superdex 200 HR 10/300). Lane P2 shows homogeneous deglycosylated LePRK1-LRR protein. Lane M, molecular-mass markers (labelled in kDa). OD, optical density.

exclusion chromatography (Superdex 200, GE Healthcare) in buffer consisting of 10 mM Tris pH 8.0, 100 mM NaCl. In the cloning procedure described above, the signal peptide (residues 1–20) was removed, resulting in release of the mature LePRK1-LRR protein (residues 21–251) from the intracellular space. The expressed and purified protein therefore contains residues 21–251 of LePRK1-LRR. For protein crystallization, the purified protein was concentrated to about 2.5 mg ml^{−1}.

2.3. Deglycosylation assay

A deglycosylation assay was performed by incubating 100 µg of the LePRK1-LRR protein with 4.0 µl PNGase F (2000 U; New England Biolabs) in 100 mM sodium chloride buffer pH 8.0. The reaction mixture (200 µl) was incubated at 37°C for 24 h; the reaction was then stopped by cooling to −80°C and the mixture was stored at this temperature prior to analysis.

2.4. Chemical cross-linking

The protein samples were incubated with suitable concentrations of the cross-linker ethylene glycol bis(sulfosuccinimidyl succinate) (EGS) for 30 min at room temperature. The reaction was stopped by the addition of 1 M Tris–HCl pH 8.0 to a final concentration of 20 mM, followed by an additional incubation for 15 min¹.

2.5. Crystallization, data collection and processing

Crystallization of the LePRK1-LRR protein was performed by the hanging-drop vapour-diffusion method. Drops were set up with 1 µl protein plus 1 µl reservoir solution at 18°C. Diffraction-quality crystals were obtained in a buffer consisting of 0.1 M Tris–HCl pH 7.0, 0.1 M ammonium acetate, 30%(w/v) polyethylene glycol (PEG) 4000. Crystals grew to their maximum size (~0.4 × 0.1 × 0.1 mm) within 3 d. For data collection, the crystals were equilibrated in a cryoprotectant buffer consisting of reservoir buffer plus 20%(v/v) glycerol. The diffraction data set was collected on beamline BL17U1 at the Shanghai Synchrotron Radiation Facility (SSRF) using an

Table 1

Data-collection and processing statistics.

Values in parentheses are for the outermost shell.

Space group	C2
Unit-cell parameters (Å, °)	$a = 136.53$, $b = 56.01$, $c = 62.93$, $\alpha = 90.00$, $\beta = 108.99$, $\gamma = 90.00$
Wavelength (Å)	0.98
Unique reflections	12216
Observed reflections	36737
Oscillation angle (°)	1
No. of frames	180
Matthews coefficient (Å ³ Da ^{−1})	2.28
Solvent content (%)	45.97
Resolution (Å)	30.00–2.75 (2.80–2.75)
$R_{\text{merge}}^{\dagger}$	0.078 (0.435)
$\langle I/\sigma(I) \rangle$	16.7 (3.8)
Completeness (%)	96.8 (98.6)
Multiplicity	3.0 (3.0)

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the average over all observations of reflection hkl .

ADSC Q315 CCD detector. The data were processed using the *HKL*-2000 suite (Otwinowski & Minor, 1997). The LePRK1-LRR crystals belonged to space group C2, with two protein molecules per asymmetric unit.

3. Results and discussion

LePRK1-LRR contains a total of four cysteines, which can form two disulfide bonds. Failure to express the LePRK1-LRR protein in *Escherichia coli* was likely to result from the existence of two disulfide bonds and some glycosylation sites. Consistent with a glycosylated LePRK1-LRR, the protein migrated as a doublet when assayed by SDS-PAGE (Fig. 3). Despite its heterogeneity, the protein was successfully crystallized using 0.1 M Tris–HCl pH 7.0, 0.1 M ammonium acetate, 30%(w/v) PEG 4000 by the hanging-drop method (Fig. 4a). The diffraction data were processed using the *HKL*-2000 suite (Otwinowski & Minor, 1997). The crystals diffracted X-rays to a resolution of 2.75 Å (Fig. 4b) and belonged to space group C2, with unit-cell parameters $a = 136.53$, $b = 56.01$, $c = 62.93$ Å, $\beta = 108.99^\circ$. Data-collection and processing statistics are summarized in Table 1.

¹ Supplementary material for this paper is available from the IUCr electronic archives (Reference: NO5039).

(a)

(b)

Figure 4
(a) LePRK1-LRR crystals. (b) An X-ray diffraction image of an LePRK1-LRR crystal.

We built a model using the data for the LRR domain and successfully solved the structure by molecular replacement.

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