Crystallization and preliminary X-ray crystallographic analysis of the extracellular domain of LePRK2 from *Lycopersicon esculentum*

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The tomato (*Lycopersicon esculentum*) pollen-specific receptor kinase 2 (LePRK2) is a member of the large receptor-like kinase (RLK) family and is expressed specifically in mature pollen and pollen tubes in *L. esculentum*. Like other RLKs, LePRK2 contains a characteristic N-terminal leucine-rich repeat (LRR) extracellular domain, the primary function of which is in protein–protein interactions. The LePRK2 LRR is likely to bind candidate ligands from the external environment, leading to a signal transduction cascade required for successful pollination. LePRK2-LRR was purified using an insect-cell secretion expression system and was crystallized using the vapour-diffusion method. The crystals diffracted to a resolution of 2.50 Å and belonged to space group *I*₄22, with unit-cell parameters $a = b = 93.94$, $c = 134.44$ Å and one molecule per asymmetric unit.

1. Introduction

Receptor-like kinases (RLKs) make up a large group of plant and animal transmembrane receptor proteins with cytoplasmic serine/threonine kinase domains and divergent extracellular domains. One of the largest subfamilies among the RLKs in plants, the LRR-RLKs, contains versatile structural motifs (~20–30 amino acids) composed of tandem arrays with two or more leucine repeats. When folded, these extracellular leucine-rich repeat (LRR) domains form a horseshoe-shaped solenoid structure with N-terminal and C-terminal caps. LRRs are found in many kinds of proteins with unrelated functions and therefore participate in many diverse functions and processes. In particular, plant-based LRRs mediate the binding of proteinaceous signalling molecules and provide a versatile structural platform for protein–protein interactions (Enkhbayar *et al.*, 2004; Kobe & Deisenhofer, 1994; Kobe & Kajava, 2001; Shiu & Bleecker, 2001).

Many identified LRR-RLKs play important roles in plant growth and development. Genetic evidence has shown that the LRR-RLK protein CLAVATA1 (CLV1) from the genus *Arabidopsis* functions to maintain stem-cell populations in the shoot apical meristem (Trotochaud *et al.*, 1999, 2000). Excess microsporocytes 1/extra sporogenous (EMS1/EXS) is a type of LRR-RLK protein which is thought to regulate the developmental fate of neighbouring tapetal cells by binding to the small extracellular protein tapetum determinant 1 (TPD1; Yang *et al.*, 2003, 2005). Additionally, the LRR-RLK protein FLS2 plays an important role in plant defence responses (Chinchilla *et al.*, 2006; Zipfel, 2008). Also, *multiple sporocytes 1* (*msp1*) encodes an LRR-RLK protein in rice, and *msp1* mutants caused male sterility (Zhao *et al.*, 2008). Furthermore, the LRR-RLK protein brassinosteroid-insensitive 1 (BRI1) mediates brassinosteroid perception, development and innate immunity (Belkhadir & Chory, 2006; She *et al.*, 2011).

The tomato (*Lycopersicon esculentum*) pollen-specific receptor kinases 1 (LePRK1) and 2 (LePRK2) are pollen-specific RLKs that are found in pollen grains and pollen tubes in tomato (Kim *et al.*, 2002; Muschietti *et al.*, 1998). The two RLKs perform different functions in pollination events. In mature pollen, LePRK1 and

LePRK2 may interact with each other to form a protein complex of nearly 400 kDa. Studies have shown that the intracellular kinase domain of LePRK2 (but not of LePRK1) is required for complex formation (Wengier *et al.*, 2003; Zhang, Liang *et al.*, 2008). Pollen-tube growth and pollen germination are hampered by a reduction in the expression levels of LePRK2 (Zhang, Wengier *et al.*, 2008).

Full-length LePRK2 protein contains 642 amino acids (nearly 71 kDa) with an extracellular domain (LRR; residues 1–219), a single transmembrane segment (220–248) and a cytoplasmic kinase domain (249–642) (Fig. 1). The LRR is comprised of 219 amino acids (nearly 23 kDa), including a 24-amino-acid signal peptide (residues 1–24), a hydrophobic N-terminal sequence (LRRNT; 25–83), five LRRs in tandem (84–198) and a hydrophobic C-terminal cap (LRRCT; 199–219) (Fig. 1). The extracellular domain of LePRK2 (LePRK2-LRR), which contains five leucine-rich repeats, may be the receiving region for signals from the extracellular environment. Several small extracellular proteins are purported ligands involved in interactions with the extracellular domains of LePRK2 (Tang *et al.*, 2002, 2004; Guyon *et al.*, 2004). Although both LePRK2 and the purported ligands are expressed specifically in pollen itself, the theory is reasonable as in autocrine signalling systems both the ligand and its receptor are produced by the same cell (DeWitt *et al.*, 2001). These external interactions lead to activation of the signalling pathway *via* phosphorylation of the intracellular LePRK2 kinase domain (Muschiatti *et al.*, 1998). Previous studies have shown that the amount of LePRK2 significantly increases after pollen germination (Wengier *et al.*, 2003). In addition, the dephosphorylation and disruption of the LePRK complexes may be mediated by the plant style, suggesting a role for LePRK2 in signal-related regulation of the pistil response (Wengier *et al.*, 2003).

Studies using transgenic plants with decreased LePRK2 function have shown the following three effects: (i) the pollen germination rate and duration were decreased, (ii) the growth rate of the pistil pollen tube was decreased and (iii) the pollen tubes were unresponsive to extracellular pro-growth signals in style extracts derived from pistils (Zhang, Wengier *et al.*, 2008). Taken together, these data demonstrated that LePRK2 plays critical roles in the regulation of pollen-tube growth and pollen germination.

Many studies have revealed that during all stages of pollen-tube growth, LePRK2 mediates a variety of roles in pollination *via* the interaction of distinctive likely ligands (Tang *et al.*, 2002, 2004; Wengier *et al.*, 2003; Zhang, Wengier *et al.*, 2008). However, the detailed molecular mechanisms of how LePRK2 perceives signals from the extracellular environment are still poorly understood. Therefore, elucidation of the three-dimensional LePRK2-LRR structure may help to provide deep and extensive insights to decipher its endogenous ligands. For this purpose, LePRK2-LRR was expressed in an insect-cell secretion expression system and crystallized.

2. Materials and methods

2.1. Molecular cloning

Total RNA was purified from *L. esculentum* using an EZNA Total RNA Kit I (Omega). Reverse-transcriptase PCR was carried out with a OneStep RT-PCR kit (Omega). The cloning primers for LePRK2-LRR (amino acids 1–219) were as follows: forward primer, PRK2-*Bam*HI-F (5'-CGGGATCCATGTCACAAAAAAC-3'); reverse primer, PRK2-219-*Xho*I-6His-R (5'-CCGCTCGAGatggtgatggtgatggtgCTTATTGCATGACTTTGC-3'). The PCR product was digested

with *Bam*HI and *Xho*I enzymes and then cloned into pFastBac vector.

2.2. Protein expression and purification

Expression of LePRK2-LRR (amino-acid residues 1–219) from tomato (*L. esculentum*) was carried out using a baculovirus insect-cell system with an engineered C-terminal 6×His tag and a modified N-terminal haemolysin peptide and was cloned into a pFastBac1 vector (Invitrogen). The protein was expressed in *Spodoptera frugiperda* (Sf21) insect cells by incubation at 28°C. 1 l of 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 harvested 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. Soluble protein was obtained and the eluted protein was

Figure 1
Secondary structure of LePRK2-LRR. The limits of the domains (amino-acid numbers) are indicated. SP, 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
Size-exclusion chromatographic analysis (Superdex 200 HR 10/300) of LePRK2-LRR protein (red curve) and 40 kDa molecular-mass marker for size-exclusion chromatography (sample S, black curve). The elution volume of the protein is indicated. Lane deG, deglycosylated LePRK2-LRR protein. Lane M, molecular-weight markers (labelled in kDa).

Table 1

Data-collection statistics for LePRK2-LRR.

Values in parentheses are for the outermost shell.

Space group	<i>I</i> 4 ₁ 22
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 93.94, <i>c</i> = 134.44, <i>α</i> = <i>β</i> = <i>γ</i> = 90.00
Wavelength (Å)	0.98
Oscillation angle (°)	1
No. of frames	180
Matthews coefficient (Å ³ Da ^{−1})	3.37
Solvent content (%)	61.53
Unique reflections	20003
Observed reflections	162124
Resolution range (Å)	47.25–2.50 (2.59–2.50)
<i>R</i> _{merge} †	0.085 (0.538)
<i>I</i> (σ(<i>I</i>))	18.84 (18.75)
Completeness (%)	99.5 (100)
Multiplicity	8.1 (8.4)

† $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, 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*.

concentrated and further purified by size-exclusion chromatography (Superdex 200 HR 10/300, GE Healthcare) in buffer consisting of 10 mM Tris pH 8.0, 100 mM NaCl. The signal peptide (residues 1–24) was removed, resulting in the release of mature LePRK2-LRR protein (25–219) from the intracellular space. Therefore, LePRK2-LRR protein consisting of residues 25–219 may be present as the purified protein. Protein purification was performed at 4°C. Protein samples from every fraction were applied to SDS–PAGE and were verified by Coomassie Blue staining (Fig. 2).

Size-fractionation studies indicate that LePRK2 is present as two bands. One is nearly 23 kDa, consistent with a monomer in size. As a result of *N*-linked glycosylation of the LePRK2-LRR fragment, the other is approximately 27 kDa. For crystallization, the purified protein was concentrated to nearly 2.5 mg ml^{−1}.

2.3. Deglycosylation assay

A deglycosylation assay was performed by incubating 100 µg of the LePRK2-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 stopped by cooling to −80°C and the reaction mixture was stored at this temperature prior to analysis.

2.4. Crystallization, data collection and processing

Crystals of LePRK2-LRR were generated by the hanging-drop vapour-diffusion method. The drops were set up with 1 µl protein solution plus 1 µl reservoir solution at 18°C. Diffraction-quality crystals were obtained under the following conditions: 0.1 M Tris–HCl pH 6.5, 0.2 M ammonium sulfate, 25% (w/v) polyethylene glycol (PEG) 8000. The crystals grew to their maximum size (~0.5 × 0.1 × 0.1 mm) within 60 d. For data collection, the crystals were equilibrated in a cryoprotectant buffer consisting of reservoir buffer plus 20% (v/v) glycerol. All diffraction data sets were collected on beamline BL17U1 at the Shanghai Synchrotron Radiation Facility (SSRF) using an ADSC Q315 CCD detector. The data were processed using the HKL-2000 software suite (Otwinowski & Minor, 1997). The crystals of LePRK2-LRR belonged to space group *I*4₁22, with one protein molecule per asymmetric unit.

3. Results and discussion

In this study, we successfully expressed the extracellular domain of LePRK2 in an insect-cell secretion expression system. The LePRK2-LRR protein was purified by affinity chromatography and size-exclusion chromatography.

Consistent with glycosylated LePRK2-LRR, the protein migrated as a doublet when assayed by SDS–PAGE (Fig. 2). Despite this heterogeneity, LePRK2-LRR crystals could be generated by the hanging-drop vapour-diffusion method at 22°C using a reservoir solution consisting of 0.1 M Tris–HCl pH 6.5 (Fig. 3), 0.2 M ammonium sulfate, 25% (w/v) PEG 8000. X-ray diffraction data were collected to 2.50 Å resolution (Fig. 3). A total of 162 124 measured reflections were merged into 20 003 unique reflections, giving an *R*_{merge} of 0.085 (0.538 in the outermost shell) and a completeness of 99.5%. The space group was determined to be *I*4₁22. The unit-cell

(a)

(b)

Figure 3

(a) LePRK2-LRR crystals. (b) An X-ray diffraction image of an LePRK2-LRR crystal.

parameters were $a = b = 93.94$, $c = 134.44$ Å. Data-collection and processing statistics are summarized in Table 1. Our data indicate that deglycosylation is dispensable for crystallization of the glycosylated protein (Fig. 2). The most similar homologue of known structure is the LRR-RLK protein SERK1 (PDB entry 4lsx; Santiago *et al.*, 2013), which has a sequence identity of 31% and a sequence similarity of 50%. Using SERK1 as a model, we built a model using the data of the LRR domain and successfully solved the structure by molecular replacement. Because the signal peptide (residues 1–24) was removed after the protein had been expressed, folded and secreted from the intracellular space, only the fragment 25–219 is present in this structure.

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