Ectodomain of plasmodesmata-localized protein 5 in *Arabidopsis*: expression, purification, crystallization and crystallographic analysis

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Plasmodesmata-localized protein 5 (PDLP5) is a cysteine-rich receptor-like protein which is localized on the plasmodesmata of *Arabidopsis thaliana*. Overexpression of PDLP5 can reduce the permeability of the plasmodesmata and further affect the cell-to-cell movement of viruses and macromolecules in plants. The ectodomain of PDLP5 contains two DUF26 domains; however, the function of these domains is still unknown. Here, the ectodomain of PDLP5 from *Arabidopsis* was cloned and overexpressed using an insect expression system and was then purified and crystallized. X-ray diffraction data were collected to 1.90 Å resolution and were indexed in space group *P*1, with unit-cell parameters $a = 41.9$, $b = 48.1$, $c = 62.2$ Å, $\alpha = 97.3$, $\beta = 103.1$, $\gamma = 99.7^\circ$. Analysis of the crystal content indicated that there are two molecules in the asymmetric unit, with a Matthews coefficient of $2.51 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 50.97%.

1. Introduction

Cell-to-cell communication is crucial for the growth and development of multicellular organisms. Such communication can provide positional and cell-fate information to the cells (Kitagawa & Jackson, 2017). In plants, the continuity of the cytoplasm between adjacent cells is mediated by cell-to-cell junctions called plasmodesmata (PD) that allow communication, and these plasma-membrane-lined channels provide an intercellular trafficking gate for small molecules and proteins (Paultre *et al.*, 2016). Besides this, PD facilitate the interconnection of cytoplasts and the formation of the symplast (Erickson, 1986). PD are also reported to be involved in various processes related to plant systemic immunity, and their permeability is tightly linked to defence-related components (Simpson *et al.*, 2009; Lim *et al.*, 2016). Recent studies of plasmodesmata-localized proteins (PDLPs) demonstrated that they are located in the PD and are involved in the cell-to-cell movement of viruses and macromolecules in plants through regulation of PD permeability (Carella *et al.*, 2015).

PDLPs are cysteine-rich receptor-like PD-localized proteins (Thomas *et al.*, 2008). There are eight PDLP members (PDLP1–PDLP8) in *Arabidopsis*, which show structural similarities to each other. PDLPs are composed of a cytoplasmic region, a single transmembrane region and an ectodomain region containing two domains of unknown function (DUF26); the DUF26 domain commonly exists in cysteine-rich receptor-like kinases (CRKs) with the conserved cysteine motif C-X₈-C-X₂-C (Thomas *et al.*, 2008; Miyakawa *et al.*,

2009). PDLs are assumed to function as a scaffold or receptor for the movement of viral proteins through the PD (Amari *et al.*, 2010), and they may have functional redundancy owing to their similarity in structure and expression pattern. It has been reported that the overexpression of PDL1 or PDL5 enhances the deposition of callose on plasmodesmata, which in turn reduces the cell-to-cell movement of fluorescent molecules and viral movement proteins (Lee *et al.*, 2011; Wang *et al.*, 2013). PDL5 is also known to be induced by salicylic acid and pathogen effectors, and this regulation may be caused by preventing intercellular transport of the DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1) protein (Carella *et al.*, 2015).

PDL5 shares 10% sequence identity with the ginkgo antifungal protein ginkbilobin-2 (Gnk2), and the latter possesses only one DUF26 domain. The structure of Gnk2 has recently been solved and it was shown that it is composed of a compact single-domain architecture with an $\alpha+\beta$ fold, and the structure of a complex of Gnk2 with mannose identified it to be a mannose-binding lectin, which confirms its antifungal activity (Miyakawa *et al.*, 2014). However, the role of DUF26 is still poorly addressed, and as the PDL5 protein contains two DUF26 domains and is involved in PD permeability, it is worthwhile solving the structure of PDL5 to provide insight into how these two DUF26 domains are packed and into the possible molecular mechanism behind the regulation of PD permeability, as well as plant systemic immunity. In this study, we report the purification, crystallization and diffraction of the ectodomain of PDL5 from *Arabidopsis*.

2. Materials and methods

2.1. Macromolecule production

The ectodomain of PDL5 (residues 1–240; N-PDL5) fused with 6×His at the C-terminus was cloned into the vector pFastBac 1 (Invitrogen) using BamHI/XhoI restriction sites (Fig. 1*a*). The fused vector was further transformed into DH10Bac[™] competent cells to obtain the recombination plasmid, and this recombinant plasmid was then used to transfect Sf9 insect cells to generate the recombinant baculovirus.

1 l of Sf9 cells (1.5×10^6 cell ml⁻¹) was infected with 10 ml of baculovirus. The cells were grown at 300 K in the dark. After 48 h of infection by the baculovirus, the cells were harvested by centrifugation at 4000 rev min⁻¹ for 30 min. The harvested cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, 150 mM NaCl pH 8.0), sonicated on ice for 99 cycles (sonication for 10 s, rest for 10 s) and then centrifuged at 12 500 rev min⁻¹ for 25 min. The protein was enriched from the supernatant medium using a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen). The column was washed with five column volumes of wash buffer (20 mM Tris-HCl, 150 mM NaCl pH 8.0). The protein was then eluted with elution buffer (20 mM Tris-HCl, 150 mM NaCl, 100 mM imidazole pH 8.0). The eluted fractions were subjected to size-exclusion chromatography (Superdex 75, GE Healthcare). The fractions containing N-PDL5 were pooled and concentrated to 5 mg ml⁻¹ for subsequent crystallization experiments. 12% SDS-PAGE was used to check the purity of the protein and

Figure 1

(*a*) Schematic domain construction of N-PDL5. Domains and regions are shown and labelled as follows: SP, signal peptide; DUF26, DUF26 domain; TM, transmembrane region; CT, C-terminal cytoplasmic region. The ectodomain region (residues 1–240) constructed into the vector is highlighted in the rectangle (grey dashed lines). The N-terminal and C-terminal residues of each region are labelled on the right. (*b*) Purification of recombinant N-PDL5. Size-exclusion chromatography of N-PDL5 on a Superdex 75 10/300 GL column (GE Healthcare). The main peak at 16 ml corresponds to a molecular weight of approximately 35 kDa, which corresponds to a monomer. The inset shows SDS-PAGE analysis of the fractions from gel filtration. Lane M, marker; lanes 1–7, fractions from the main peak from gel filtration.

Table 1
Macromolecule-production information.

Source organism	<i>A. thaliana</i>
DNA source	cDNA
Forward primer†	CGCGGATCCATGATCAAGACAAAGACGAC
Reverse primer†	CCGCTCGAGTCAGTGGTGGTGGTGGTGGTG CTGACGCGCGTGACTACCAC
Expression vector	pFastBac 1 (Invitrogen)
Expression host	Sf9 insect cells
Complete amino-acid sequence of the construct produced	VFYCFRNSFVIKKPINIPDYSYRPTIGRGS MAFKSIAVLSACIIVGSALGPGGSPTDN YIYAVCSPAKFSPSSGYETNLSLLSSF VTSTAQTRYANFTVPTGKPEPTVTVYGI YQCRGDLDPACSTCVSSAVAQVGALCS NSYSGFLQMENCLIRYDNKSFLGVQDKT LILNKGQPMFNDQDALTASDVIGSL GTGDGSYRTGGNGNVQGVACQSGDLSTS QCQDCLSDAIGRLKSDCGMAQGGYVYLS KCYARFSVGGSHARQHHHHHH

† The BamHI and XhoII restriction sites are underlined.

the homogeneity of the protein was determined to be >95% (Fig. 1*b*). Protein-production information is summarized in Table 1.

2.2. Crystallization

Conditions for the crystallization of N-PDLP5 were initially screened by the hanging-drop vapour-diffusion method with drops consisting of 1 µl protein solution mixed with an equal volume of reservoir solution in 16-well plates at 289 K using commercial screening kits from Hampton Research, USA. Each crystallization drop was equilibrated against 200 µl reservoir solution. Initial microcrystals were obtained after 3 d from three conditions: (i) 0.1 M sodium citrate tribasic dihydrate pH 5.0, 10%(*w/v*) polyethylene glycol 6000, (ii) 0.1 M bis-tris propane pH 9.0, 30%(*w/v*) polyethylene glycol 6000 and (iii) 0.1 M citric acid pH 3.5, 28%(*w/v*) polyethylene glycol 8000. Unfortunately, the crystals grown in all three of the conditions mentioned above diffracted poorly; the crystallization conditions were therefore further optimized by adjusting the concentration of precipitant, the crystallization temperature and the buffer pH, and by screening using the

Figure 2
Crystals of N-PDLP5 obtained by the hanging-drop method at 293 K in 0.1 M citric acid pH 3.5, 0.1 M sodium citrate tribasic dihydrate pH 5.5, 28%(*w/v*) polyethylene glycol 8000. The scale bar is 0.2 mm in length.

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	16-well plates
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	5
Buffer composition of protein solution	20 mM Tris-HCl, 150 mM NaCl pH 8.0
Composition of reservoir solution	0.1 M citric acid pH 3.5, 0.1 M sodium citrate tribasic dihydrate pH 5.5, 28%(<i>w/v</i>) polyethylene glycol 8000
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir (µl)	200

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline BL17U1, SSRF
Wavelength (Å)	0.97884
Temperature (K)	100
Detector	ADSC Q315 CCD
Crystal-to-detector distance (mm)	180
Rotation range per image (°)	0.5
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	41.9, 48.1, 62.2
α , β , γ (°)	97.3, 103.1, 99.7
Mosaicity (°)	0.81
Resolution range (Å)	50.00–1.90 (1.93–1.90)
Total No. of reflections	161426 (7475)
No. of unique reflections	33880 (1699)
Completeness (%)	92.3 (90.3)
Multiplicity	4.8 (4.4)
$\langle I/\sigma(I) \rangle$	46.1 (13.5)
<i>R</i> _{int}	0.074 (0.391)
Overall <i>B</i> factor from Wilson plot (Å ²)	21.4
<i>CC</i> _{1/2}	0.984 (0.893)

Additive and Detergent Screens from Hampton Research to improve the crystal quality. After optimization, the final diffraction-quality crystals with a maximum length of 700 µm were obtained using a solution consisting of 0.1 M citric acid pH 3.5, 0.1 M sodium citrate tribasic dihydrate pH 5.5, 28%(*w/v*) polyethylene glycol 8000 (Fig. 2 and Table 2). We also performed peptide mass fingerprinting analysis of the washed crystals to confirm that the crystal was of N-PDLP5 (Supplementary Table S1).

2.3. Data collection and processing

The loop-mounted crystals were directly flash-cooled in liquid nitrogen, and X-ray diffraction data collection was performed under cryogenic conditions at 100 K on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF), People’s Republic of China. A total rotation range of 360° was covered using 0.5° oscillation and 1 s exposure per frame. The wavelength of the synchrotron X-ray beam was 0.97884 Å, and the crystal-to-detector distance was set to 180 mm. The diffraction data were indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). Data-collection and processing statistics are summarized in Table 3.

Figure 3

A diffraction image from an N-PDLP5 crystal with resolution rings shown. *HKL-2000* (Otwinowski & Minor, 1997) was used to generate the image.

3. Results and discussion

The ectodomain of PDLP5 from *Arabidopsis* containing two DUF26 domains was cloned and expressed using the Bac-to-Bac[™] secreted insect expression system, and was then purified and crystallized. To date, we have collected X-ray diffraction data to 1.90 Å resolution (Fig. 3); the data were indexed in space group *P*1, with unit-cell parameters $a = 41.9$, $b = 48.1$, $c = 62.2$ Å, $\alpha = 97.3$, $\beta = 103.1$, $\gamma = 99.7^\circ$. Based on the Matthews coefficient, we propose that the asymmetric unit contains two PDLP5 molecules, with a Matthews coefficient of $2.51 \text{ Å}^3 \text{ Da}^{-1}$ and a corresponding solvent content of 50.97%. We also used *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* suite (Winn *et al.*, 2011) to calculate the self-rotation function of the N-PDLP5 crystal, and the function revealed the presence of a twofold noncrystallographic axis (Supplementary Fig. S1), suggesting that there are two PDLP5 molecules in the asymmetric unit, which is consistent with the results suggested from the Matthews coefficient. We attempted molecular replacement using the structure of GnK2 as a search model (PDB entry 3a2e; Miyakawa *et al.*, 2009). The sequence identity between N-PDLP5 and GnK2 is 24%,

but the query coverage was only 42%. Therefore, we did not obtain a satisfactory solution. We are currently attempting other molecular-replacement searches and are preparing selenomethionine-substituted and heavy-atom derivatives in order to obtain accurate phases using anomalous dispersion techniques.

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