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Crystallization of DIR1, a LTP2-like resistance signalling protein from *Arabidopsis thaliana*

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DIR1, a putative LTP2 protein from *Arabidopsis thaliana* implicated in systemic acquired resistance *in planta*, has been crystallized in space group $P2_12_12_1$ with one molecule per asymmetric unit. The crystals diffract to a resolution of 1.6 Å.

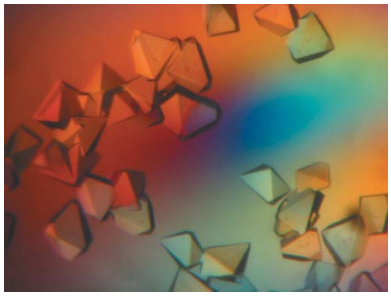
1. Introduction

Lipid-transfer proteins (LTPs) are ubiquitous lipid-binding proteins from plants. Two multigenic families have been identified, LTP1 and LTP2. Both LTP1 and LTP2 display low or no amino-acid sequence identity except for a common motif of eight cysteines involved in four disulfide bridges (Fig. 1). This motif is now considered to be the LTP signature and reflects a common helical fold stabilized by four helices. LTPs have a large central hydrophobic tunnel or cavity that allows the binding of different types of lipids and hydrophobic molecules. In *Arabidopsis thaliana*, more than 20 genes encoding LTPs have been highlighted. They are expressed or induced during a wide range of biotic or abiotic stress. These proteins have been associated with such different physiological events that their precise functions still remain to be discovered (Douliez *et al.*, 2000; Marion *et al.*, 2004). However, most of the data point to a role of these proteins in the defences of plants against their microbial predators. In particular, it has been shown that LTP1s and oomycetous elicitins compete for a plasma membrane receptor involved in the hypersensitive response (HR) and systemic acquired resistance (SAR) in *Nicotiana tabacum* (Buhot *et al.*, 2001; Blein *et al.*, 2002). This is strengthened by the observation that a mutant of *A. thaliana* defective in a gene encoding a putative LTP2, *i.e.* DIR1, was unable to develop SAR (Maldonado *et al.*, 2002). Therefore, all these studies suggest that *in planta* LTPs are probably involved in the signalling of plant defence mechanisms. DIR1 (defective in induced resistance 1) is not the signalling molecule *stricto sensu*, but could be involved in the transport of lipids that are the effective SAR signals (Maldonado *et al.*, 2002). Therefore, to understand the relationship between the biological function of DIR1 and its lipid-binding properties, we have crystallized a protein–lipid complex of DIR1.

2. Experimental

2.1. Preparation and purification

The mature DIR1 protein (gene accession No. At5g48485) from *A. thaliana* was cloned into pPICZα vector (Invitrogen) and expressed in the methylotrophic yeast *Pichia pastoris*. The yeast culture supernatant was mostly constituted of the native DIR1



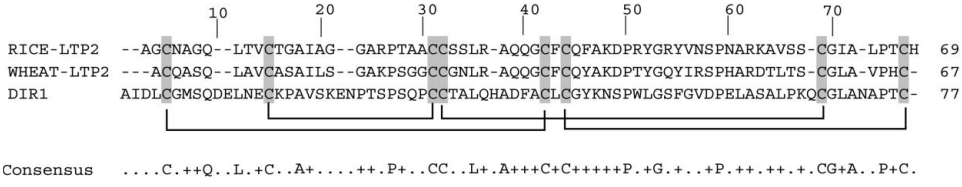


Figure 1
LTP2 alignments. The conserved cysteine residues are boxed. The numbering is given with respect to DIR1.

Table 1
Crystallographic details and statistics of diffraction data for DIR1 (all data were recorded at 100 K).

Values in parentheses are for the outer resolution shell.

		Zn			
	S	Peak	Edge	High remote	Native
Wavelength (Å)	1.700	1.2822	1.2831	1.2770	0.9800
Crystal No.	1				2
Crystal-to-detector distance (mm)	145	190			230
No. of frames ($\Delta\omega = 1^\circ$)	400	400			180
Space group	$P2_12_12_1$				
Unit-cell parameters (Å)					
a	28.236	28.240	28.207	28.231	28.345
b	48.149	48.150	48.100	48.111	48.161
c	55.009	55.010	54.994	55.026	54.542
No. of measured reflections	70319	97346 (11546)	94807 (12494)	90034 (13550)	65812 (8066)
No. of unique reflections	6423 (364)	7099 (1012)	6797 (957)	6309 (905)	10294 (1472)
Multiplicity	10.9 (4.3)	13.7 (11.4)	13.9 (12.9)	14.3 (14.0)	6.4 (5.5)
Resolution limits (Å)	55–1.8 (1.9–1.8)	15–1.82 (1.92–1.82)	54–1.85 (1.95–1.85)	54–1.9 (2.0–1.9)	54–1.60 (1.69–1.6)
Data completeness (%)	86.9 (35.1)	99.6 (100.0)	99.7 (99.6)	99.7 (99.6)	99.4 (99.8)
Mean $I/\sigma(I)$	25.5 (3.9)	31.7 (8.1)	32.8 (8.8)	29.6 (9.5)	24.9 (5.6)
$R_{\rm merge}$† (%)	7.9 (19.0)	7.3 (27.9)	7.3 (31.0)	8.0 (33.1)	6.0 (21.2)
Anomalous statistics					
Completeness (%)	86.3 (33.4)	99.7 (100.0)	99.8 (99.9)	99.8 (99.8)	–
Multiplicity	5.9 (2.3)	7.5 (6.0)	7.6 (6.9)	7.8 (7.8)	–
$\Delta_{\rm anom}$ correlation (±)	−0.05 (−0.01)	0.522 (0.206)	0.237 (0.156)	0.316 (0.061)	–

† $R_{\rm merge} = \sum_{\mathbf{h}}\sum_i |\langle I(\mathbf{h})\rangle - I(\mathbf{h})| / \sum_{\mathbf{h}}\sum_i I_i(\mathbf{h})$, where $I_i(\mathbf{h})$ is the ith observation of reflection $\mathbf{h}$.

protein. It was first centrifuged and then purified using the following three-step method.

Firstly, the *Pichia* supernatant was concentrated and desalted using adsorption chromatography (XAD7 resin, Amberlite). Secondly, the protein was eluted using an acetonitrile gradient (50% acetonitrile, 0.1% TFA). Thirdly, the resulting material was loaded onto a High Prep SP-XL column (Amersham) pre-equilibrated with 50 m*M* sodium acetate pH 3.5 (elution took place using a 1 *M* NaCl gradient at pH 3.5). The native protein was finally purified using reverse-phase chromatography. The homogeneity and the purity of the protein were checked by mass spectrometry.

2.2. Crystallization

The protein was first incubated with monostearoyl phospatidylcholine as a putative substrate in a ratio of 1:1.5 for 1 d at 277 K. Preliminary screenings were performed using a Cybi-Disk robot system with Hampton Research Crystal Screen I and II kits. 96-Condition plates were employed with a 100 µl reservoir and 0.5 µl drops composed of 50% reservoir solution and 50% protein–complex solution (20 mg ml^{-1}). The best hits leading to crystals always contained zinc salts and PEG MME at acidic pH. Optimization of these conditions took place in standard Linbro plates. The final optimized conditions for routine preparation were 0.08 *M* acetate buffer pH 6.0 containing 0.02 *M* $ZnSO_4$ and 20–25% PEG 600 monomethyl ether as the reservoir solution and a hanging drop made up of 1 µl of protein at 20 mg ml^{-1} in 0.02 *M* $ZnSO_4$ pH 6.0 mixed with 1 µl reservoir solution. Crystals grow to their final size (Fig. 2) in about a month.

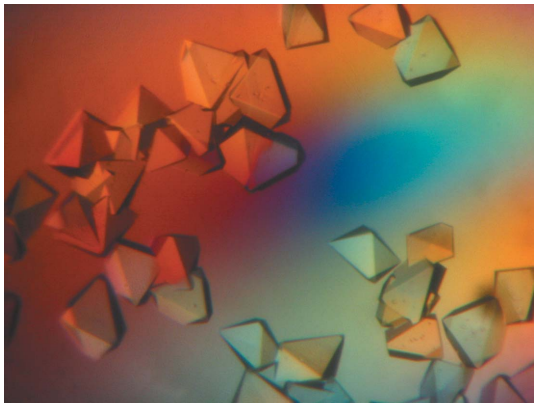


Figure 2
Crystals of DIR1. Maximum size is 0.2 mm.

2.3. Data collection

Data collection took place at ESRF, Grenoble on beamline ID-29 using an ADSC CCD detector. Crystals were frozen in liquid nitrogen. No cryoprotectant was used owing to the high polyethylene glycol content. Three different strategies were applied. An initial highly redundant data collection (400° rotation) was recorded at 1.9 Å in order to exploit the anomalous signal of sulfur, which was present at a high level (eight cysteines forming four disulfide bridges plus one methionine in 77 residues). A second round of three data collections took place using the same crystal at the Zn *K* edge (for MAD techniques) and a final data collection was performed at the highest possible resolution with a fresh crystal at a shorter wavelength. The data collections and their diffraction statistics are reported in Table 1. In all cases, the diffraction extends to a limit of 1.6–1.9 Å; however, this was associated with a high mosaicity.

All data were processed using *MOSFLM*, merged with *SCALA* and finally put on an absolute scale with *TRUNCATE*, all of which are available from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The unit-cell parameters ($a = 28.23$, $b = 48.15$, $c = 55.01$ Å) and volume ($V = 74\,786$ Å^3) correspond to a Matthews coefficient of 2.32 Å^3 Da^{-1}, which accounts for one molecule in the asymmetric unit and a solvent content of 47%.

Preliminary attempts to solve the structure by molecular replacement using the wheat (PDB code 1tuk; Hoh *et al.*, 2005), rice (PDB code 1l6h; Samuel *et al.*, 2002) or other LTP2 coordinates (PDB code 2alg; Pasquato *et al.*, 2006) failed, probably because of the low identity (29–26%) to DIR1. We expect ordered Zn atoms in the

crystal structure of DIR1 and are therefore attempting to solve the structure using the MAD data at the Zn *K* edge.

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