Cloning, overexpression, purification and preliminary X-ray analysis of the protein kinase domain of enhanced disease resistance 1 (EDR1) from *Arabidopsis thaliana*

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Enhanced disease resistance 1 is a member of the Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK) family that negatively regulates disease resistance, ethylene-induced senescence and programmed cell death in response to both abiotic and biotic stresses. A catalytically inactive form of the EDR1 kinase domain was successfully cloned, expressed, purified and crystallized. Crystallization was conducted in the presence of the ATP analogue AMP-PNP. The crystals belonged to space group $P3_221$ and contained two molecules in the asymmetric unit. The crystals diffracted X-rays to 2.55 Å resolution.

1. Introduction

Plants, like mammals, are faced with a wide range of attacking pathogens against which they have developed elaborate defence mechanisms. Since plants lack a mobile cell defence characteristic of an adaptive immune system, they rely on innate defences such as protective cell walls and so-called resistance genes. In the event of pathogen attack, plants detect specific pathogen-related molecules to induce both local and global defence mechanisms. To this end, plants use pattern-recognition receptors (PRRs) to detect pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), thus triggering a complex set of events ranging from the primary response to the activation of hypersensitive response (HR) leading to localized programmed cell death (Jones & Dangl, 2006; de Wit, 2007; Boller & He, 2009). The hypersensitive response usually precedes the formation of global disease resistance, also known as systemic acquired resistance (SAR), which involves the expression of the antimicrobial *pr* (pathogenesis-related) genes in a salicylic acid (SA)-dependent manner in tissues distant from the original infection site (Fu & Dong, 2013). Alongside SAR, plants also employ another similar form of systemic immunity, called induced systemic resistance (ISR), to achieve long-term protection against attacking microorganisms. ISR is independent of SA signalling but is instead dependent on the activity of two other phytohormones: ethylene and jasmonic acid (Pieterse *et al.*, 1998). The cross-talk between the different plant hormone pathways is important both for the local and global defence mechanisms and provides the means to fine-tune the associated defence responses (Pieterse *et al.*, 2009).

The gene encoding for enhanced disease resistance 1 (EDR1) was first discovered in loss-of-function mutants of *Arabidopsis thaliana* showing enhanced disease resistance to *Erysiphe cichoracearum*, the causative agent of powdery mildew (Frye & Innes, 1998). EDR1 is a serine/threonine-protein kinase that belongs to the superfamily of Raf-like mitogen-activated protein kinase kinase kinases (MAPKKKs). It shares highest similarity (43% identity) with another *A. thaliana* serine/threonine kinase of the same class called constitutive triple response 1 (CTR1; Frye *et al.*, 2001). EDR1 acts as a negative regulator of disease resistance and programmed cell death in an SA-dependent manner (Frye *et al.*, 2001; Tang *et al.*, 2005). The *edr1* mutant plant also shows enhanced drought-induced growth

Table 1

Macromolecule-production information.

Source organism	<i>A. thaliana</i>
DNA source	<i>A. thaliana</i>
Forward primer	TACTTCCAATCCAATGCCGATGATGATGTTGGTGAATGT-GAAATTC
Reverse primer	TTATCCACTTCCAATGTTATTGTGGTGTAGGAAGTACAAGCC-GGTTC
Cloning vector	pMCSG7
Expression vector	pMCSG7
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	DDADVGECEIPWNDLVIAERIGLSYGEVYHADWHGTEVAVK-KFLDQDFSGAALAEFRSEVRIMRRLRHPNVVFFLGAVTRP-PNLSIVTEFLPRGSLYRILHRPKSHIDERRRIKMLDVM-GMNCILHTSTPTIVHRNLKTPNLLVDNNWNVKVGDFGLSRL-KHNTFLSSKSTAGTPEWMAPEVLNREPSNEKCDVYSFGVILWELATLRLPWRGMNPMQVVGAVGFQNRRLLEIPKELDPVV-GRITILECWQTDPNLRPSFAQLTEVLKPLNRLVLPPTQ

inhibition, enhanced ethylene-induced senescence and enhanced sensitivity to the plant hormone abscisic acid (ABA), therefore linking EDR1 to at least two other plant hormone signalling pathways (Frye *et al.*, 2001; Tang *et al.*, 2005). Ubiquitin-mediated protein degradation has been suggested as a control mechanism in the EDR1-mediated signalling pathway based on the involvement of F-box protein ORE9 in the drought-induced growth inhibition and ethylene-induced senescence phenotypes (Tang *et al.*, 2005). EDR1 is also known to interact with KEG (KEEP ON GOING), a RING-finger E3-ubiquitin ligase that negatively regulates the protein levels of the transcription factor ABI5 (abscisic acid-insensitive 5) (Wawrzynska *et al.*, 2008; Gu & Innes, 2011). The interaction of EDR1 with KEG was shown to be important for EDR1 function since a missense mutation in *keg* suppresses all EDR1-mediated phenotypes (Wawrzynska *et al.*, 2008). It has been proposed that KEG could serve as a substrate for EDR1 and that EDR1 might therefore regulate its downstream components by KEG-mediated ubiquitination (Gu & Innes, 2011).

EDR1 is known to have kinase activity and to autophosphorylate *in trans* (Tang & Innes, 2002). Structural characterization of the EDR1 kinase domain will provide further clues to its regulatory and activation mechanisms.

2. Materials and methods

2.1. Cloning

The C-terminal kinase domain of EDR1 (EDR1-kd, residues 655–933) was amplified by PCR from a cDNA library obtained from the *Arabidopsis* Biological Research Center at Ohio State University (Kieber *et al.*, 1993). The sequences of the forward and reverse primers were 5'-TACTTCCAATCCAATGCCGATGATGCA-GATGTTGGTGAATGTGAAATTC-3' and 5'-TTATCCACTTCCAATGTTATTGTGGTGTAGGAAGTACAAGCCGGTTC-3', respectively. Both primers contain the necessary extensions for ligation-independent cloning (LIC), which was used to insert the PCR product into the expression vector pMCSG7 (Eschenfeldt *et al.*, 2009). The expression vector adds an N-terminal hexahistidine tag followed by a TEV cleavage site. Because of cloning, three additional amino acids (SNA) remain at the native N-terminus after digestion with TEV protease. The correct insertion of the target gene was verified by DNA sequencing.

Mass-spectrometric analysis of EDR1-kd indicated that the sample contained several phosphorylation sites and was heterogeneous in nature. To improve the sample homogeneity, we mutated the catalytically important aspartate residue (Asp792) to asparagine using

site-directed mutagenesis PCR. The sequences for the forward and the reverse primers were 5'-CACCGACAATTGTTTC-ATCGTAATCTCAAAACACCAAACCTT-3' and 5'-AAG-GTTTGGTGTGTTTGAGATTACGATGAACAATTGTGCGGTG-3', respectively. The sequence of the final expression vector was confirmed by DNA sequencing. Table 1 summarizes the macromolecule-production information.

2.2. Expression and purification

The plasmid containing the EDR1 D792N mutant was transformed into *Escherichia coli* strain BL21 cells co-expressing chaperones DnaK, DnaJ, GrpE, ClpB, GroEL and GroES (CC4 cell lines, courtesy of A. Geerlof, Helmholtz Zentrum München). Freshly transformed cells were used to prepare 5 ml overnight pre-culture at 310 K in Luria–Bertani (LB) medium. 1 l of autoinduction medium (Studier, 2005) was inoculated with the pre-culture and incubated at 310 K until an OD₆₀₀ of between 0.6 and 0.8 was reached. After this point, incubation was continued for another 18 h at the lower temperature of 293 K. Cells were harvested by centrifugation at 5500 rev min⁻¹ in a JLA-8.1000 rotor for 25 min at 277 K. The cell pellets were resuspended in lysis buffer [50 mM HEPES pH 7.0, 400 mM NaCl, 5% (v/v) glycerol, 5 mM MgSO₄, 0.1% (w/v) CHAPS], supplemented with EDTA-free protease inhibitors (cComplete ULTRA Tablets, Mini, Roche) and 10 µg ml⁻¹ DNase. The resuspended cells were lysed by sonication on ice for two cycles of 4 min. The lysate was centrifuged at 19 000 rev min⁻¹ in an SS-34 rotor at 277 K for 30 min.

Isolation of His-tagged EDR1 D792N mutant followed standard purification procedures. The supernatant was filtered through a 0.22 µm membrane and loaded onto 2 ml Ni-NTA agarose beads (Invitrogen) in a gravity-flow column. Prior to this, the beads had been equilibrated against 25 mM NiSO₄ in water and then against buffer A [50 mM HEPES pH 7.0, 400 mM NaCl, 5% (v/v) glycerol, 5 mM MgSO₄]. After sample loading, the column was first rinsed with 10 column volumes (CV) of buffer A with 6% buffer B (buffer A with 500 mM imidazole) followed by 5 CV of buffer A with 10% buffer B. Bound protein was eluted in four consecutive steps with 15, 20 and 60% buffer B in buffer A and finally with buffer B (75–500 mM imidazole). 2 CV of buffer was used in each elution step.

Fractions containing the target protein were pooled based on SDS-PAGE (Bio-Rad Mini-PROTEAN Tetra Cell). Purified TEV protease was added in a molar ratio of 1:10 to cleave the His tag. Digestion with TEV protease was carried out overnight at 277 K in a dialysis bag equilibrating against buffer C [50 mM HEPES pH 7.0, 400 mM NaCl, 5% (v/v) glycerol, 5 mM MgSO₄, 1 mM DTT, 1 mM EGTA]. The dialyzed protein was then loaded onto the gravity-flow column again with 2 ml Ni-NTA beads in order to remove His-tagged TEV protease and uncleaved material. Tag-free protein was concentrated using Corning Spin-X UF centrifugal concentrator with a 10 kDa cutoff (Corning Incorporated Life Sciences, Tewksbury, USA) and applied onto an SEC column (HiLoad 16/60 Superdex G75, GE Healthcare) equilibrated with buffer D [50 mM HEPES pH 7.5, 400 mM NaCl, 5% (v/v) glycerol, 5 mM MgSO₄]. Protein fractions were checked by SDS-PAGE for homogeneity. Fractions containing a pure, monomeric form of the protein were pooled and concentrated to about 10 mg ml⁻¹.

2.3. Crystallization

Prior to crystallization, AMP-PNP, a nonhydrolyzable analogue of ATP, was added to the protein solution to a final concentration of 5 mM (~15-fold molar excess). Initial crystallization experiments

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	Limbro 24-well
Temperature (K)	292
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	50 mM HEPES pH 7.5, 400 mM NaCl, 5% glycerol, 5 mM MgSO ₄ , 5 mM AMP-PNP
Composition of reservoir solution	1.2 M lithium sulfate, 10% PEG 3350, 0.1 M HEPES pH 7.8
Volume and ratio of drop	4 µl and 1:1
Volume of reservoir (µl)	500

were carried out with five commercial crystallization screens (The Classics, Classics II, PEGs, PEGs II and AmSO₄ Suites, Qiagen) at the EMBL Hamburg high-throughput crystallization facility (Mueller-Dieckmann, 2006). All initial screens were performed using the sitting-drop vapour-diffusion method at 292 K in 96-well Greiner plates. 300 nl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 50 µl of reservoir solution. After 3–4 d thin, needle-like crystals were growing in several conditions containing 1.6 M ammonium sulfate in the pH range 7.0–9.0. Manual optimization was performed on these conditions using the hanging-drop vapour-diffusion method and this resulted in rod-like crystals that diffracted X-rays to 2.5 Å resolution (Fig. 1). These crystals appeared after one week in a drop that contained 1.2 M lithium sulfate, 10% (w/v) PEG 3350, 0.1 M HEPES pH 7.8. Table 2 summarizes the crystallization procedure.

2.4. X-ray diffraction data collection and processing

Prior to data collection, a single crystal of the EDR1 D792N mutant grown in the presence of AMP-PNP was soaked for about 5 s in mother liquor augmented with 15% (v/v) glycerol as a cryoprotectant and 10 mM AMP-PNP. The crystals were then flash-cooled to 77 K in liquid nitrogen. X-ray diffraction data sets were collected on beamline P13 of PETRA III, EMBL Hamburg, Germany at 100 K with a PILATUS 6M detector. From the best diffracting crystal, a total of 800 images were collected with an oscillation range of 0.1°. The data were indexed and integrated using *XDS* (Kabsch, 2010) and scaled with *SCALA* (Winn *et al.*, 2011). Table 3 summarizes the data-collection and processing statistics.

Figure 1

Crystals of the EDR1 D792N mutant from *A. thaliana*. The largest crystal shown here is about 300 × 20 × 20 µm.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	P13, PETRA III
Wavelength (Å)	1.3858
Temperature (K)	100
Detector	PILATUS 6M
Crystal-to-detector distance (mm)	246
Rotation range per image (°)	0.1
Total rotation range (°)	80
Space group	<i>P</i> ₃ ₂ ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	90.57, 90.57, 185.9
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.083
Resolution range (Å)	92.93–2.55 (2.69–2.55)
Total No. of reflections	95586 (13054)
No. of unique reflections	28736 (4049)
Completeness (%)	97.5 (96.4)
Multiplicity	3.3 (3.2)
$\langle I/\sigma(I) \rangle$	8.6 (2.0)
<i>R</i> _{p.i.m.} (%)	6.0 (47.2)
Overall <i>B</i> factor from Wilson plot (Å ²)	47.6

3. Results and discussion

The catalytic C-terminal domain of *A. thaliana* EDR1 was successfully cloned into the expression vector pMCSG7 and the catalytically important aspartate residue (Asp792) was mutated to asparagine by site-directed mutagenesis PCR in order to produce homogeneous protein from a kinase inactive enzyme. The EDR1 D792N mutant was expressed in *E. coli* strain CC4, a derivative strain of BL21(DE3) co-expressing chaperones DnaK, DnaJ, GrpE, ClpB, GroEL and GroES. The purification was conducted using Ni-affinity and size-exclusion chromatography and yielded approximately 1.2 mg pure protein from 1 l of culture. The purity of the protein was confirmed by SDS-PAGE.

Initial crystallization experiments were performed at the EMBL Hamburg high-throughput crystallization facility (Mueller-Dieckmann, 2006) using commercially available screens. Manual optimization of several lead conditions produced well diffracting, rod-like crystals from a condition that consisted of 1.2 M lithium sulfate, 10% (w/v) PEG 3350, 0.1 M HEPES pH 7.8. These crystals diffracted to 2.50 Å resolution and a data set of 800 images was collected on the PETRA III beamline at EMBL Hamburg, Germany. The crystals belonged to space group *P*₃₂₁, with unit-cell parameters *a* = *b* = 90.57, *c* = 185.90 Å, α = β = 90, γ = 120°. Based on the calculated Matthews coefficient (Matthews, 1968) of *V*_M = 2.29 Å³ Da⁻¹, the asymmetric unit is most likely to contain three molecules with a solvent content of 46.4%. However, initial results from molecular replacement using *MOLREP* (Vagin & Teplyakov, 2010) and the crystal structure of *A. thaliana* CTR1 (Mayerhofer *et al.*, 2012) as a search model indicate the presence of two molecules in the asymmetric unit. For a protein dimer the Matthews coefficient would be *V*_M = 3.44 Å³ Da⁻¹ with a solvent content of 64.2%. We are continuing our efforts to refine the crystal structure of the EDR1 D792N mutant.

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