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Purification, crystallization and preliminary X-ray diffraction analysis of effector protein MoHrip2 from *Magnaporthe oryzae*

MoHrip2, a novel effector protein from the pathogenic fungus *Magnaporthe oryzae*, was purified and crystallized using the sitting-drop vapour-diffusion method. Native crystals and selenomethionine-labelled crystals were obtained using 2.2 M ammonium sulfate as a precipitant. A native data set was collected to 2.0 Å resolution at 100 K using an in-house X-ray source and a selenomethionine-labelled data set containing anomalous signal was collected to 1.8 Å resolution at 100 K using a synchrotron source. Based on the anomalous signal generated from the Se atom, the MoHrip2 structure was successfully solved using the single-wavelength anomalous dispersion (SAD) method.

1. Introduction

Similar to mammals, plants have several layers of defence to protect themselves from pathogenic infections including the protection of a cell wall and the innate immune system. To resist the infection of pathogens, plants have evolved a series of tactics to recognize and fight against various pathogens *via* the innate immune system (Boller & He, 2009; Mukhtar *et al.*, 2011). Plants and pathogens constantly struggle throughout the process of evolution. To infect plants, pathogens damage plant defence systems by secreting effectors such as hydrolases and virulence factors that damage the cell wall and some metabolic reactions, respectively, within cells (Misas-Villamil & Hoorn, 2008).

Two branches of the innate immune system have been reported: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is thought to be induced by pathogen-associated or microbial-associated molecular patterns (PAMPs or MAMPs), such as flagellin flg22 (Zipfel *et al.*, 2004), and is recognized by pattern-recognition receptors (PRRs), which are located on the plant cell surface (Zipfel, 2008). ETI is believed to be induced by effectors that are secreted by pathogens and that act largely inside plant cells, *e.g.* avirulence protein 2 (AVR2; Luderer *et al.*, 2002). The receptors for these effectors in plant cells are known to have nucleotide-binding (NB) and leucine-rich repeat (LRR) domains (Jones & Dangl, 2006). Some components or conservative proteins of pathogens, which are primarily beneficial for self-growth, are recognized by plant receptors and result in PTI to protect plants, and pathogens can secrete effectors to avoid or suppress PTI. In contrast, plants also evolve and form new receptors to recognize pathogen effectors and subsequently result in new PTI or ETI (Thomma *et al.*, 2011). These types of struggles are the reason that the two kingdoms constantly co-evolve (Boller & He, 2009; Jones & Dangl, 2006).

Effectors from different pathogens may have a similar mechanism for activating defence systems, particularly those with similar structures (Misas-Villamil & Hoorn, 2008). In recent years, some three-dimensional structures of effectors, such as *Pyrenophora tritici-repentis* (Ptr) ToxA (Sarma *et al.*, 2005) and a necrosis and ethylene-inducing peptide 1 (Nep1)-like protein (NLP; Ottmann *et al.*, 2009), have been reported. The structures of effectors and their receptors are now being elucidated and some structures of complexes have recently been reported, such as a chitin oligomer with the ectodomain of chitin elicitor receptor kinase 1 of *Arabidopsis* (AtCERK1-ECD; Liu *et al.*, 2012). Therefore, the determination of the structures of these factors contributes to classification studies and

provides insight into their detailed mechanism for the regulation of plant immune responses.

Our laboratory isolated a novel effector protein from the pathogenic rice fungi *Magnaporthe oryzae*. This protein was analysed by *de novo* sequencing and was named MoHrip2. The *mohrip2* gene (GenBank accession No. JQ81555.1) consists of 459 bp encoding 152 amino-acid residues with a theoretical molecular weight of 16.25 kDa. Our experiments demonstrated that crude or recombinant MoHrip2 was able to trigger the hypersensitive response (HR) and could also activate the expression of some genes with regard to the tobacco cell defence system and the accumulation of NO and callose. These processes are well known early defence responses that are activated during the plant pathogen infection process (Garcia-Brugger *et al.*, 2006). However, the receptors and detailed mechanism for MoHrip2 activation are unclear.

To study the molecular mechanism of the effector protein MoHrip2 at the structural level, recombinant MoHrip2 was expressed and crystallized. A high-resolution native data set was successfully collected using an in-house X-ray source. Because there was no homologous structure for solving the phase problem, a MoHrip2 selenomethionine derivative was expressed and crystallized, and a high-resolution data set containing an anomalous selenium-atom signal was successfully collected using a synchrotron source.

2. Materials and methods

2.1. Cloning and point mutation

The full-length *mohrip2* gene consists of 459 bp encoding 152 amino-acid residues. As a secretory protein, the first 18 amino-acid residues are a signal peptide, which was predicted by the SignalP 3.0 server (Bendtsen *et al.*, 2004). The sequence without the signal peptide was cloned into the pET-30 TEV/LIC vector [reconstructed from the pET-30a(+) vector (Novagen) by the Liu Laboratory in which a thrombin recognition site, an enterokinase recognition site and an S-tag were displaced by the TEV protease recognition site and LIC sequence] downstream of a 6×His tag. The amplification of the sequence was performed by polymerase chain reaction (PCR) using the pMD R 18-T simple vector, which contained the full-length *mohrip2* gene (isolated by our laboratory), as a template. The forward primer was 5'-TACTTCCAATCCAATGCCAGCAGGC-TATCGTCCACAAC-3' and the reverse primer was 5'-TTATC-CACTTCCAAGCTATTAGGCGCAGAGGGTAGCC-3'. The PCR program was as follows: initial denaturation for 6 min at 367 K, 30 cycles of 367 K for 40 s, 331 K for 40 s and 345 K for 40 s, and a final extension for 10 min at 345 K. The PCR products were purified with

the AxyPrep DNA Gel Extraction Kit (Axygen) and then cloned into the vector using ligation-independent cloning (Aslanidis & Jong, 1990).

To ensure an adequate diffraction signal for the Se atom, more than two selenomethionines were needed in one molecule based on the length of MoHrip2 (we were unable to determine phases using the signal from one selenomethionine). However, only one methionine exists in the 19–152 amino-acid residue sequence; thus, we needed to introduce another methionine. Based on the analysis for the prediction of the MoHrip2 secondary structure, we chose the leucine located in the 78th codon as the mutation site; the mutant protein was named MoHrip2-L78M. Based on the QuikChange site-directed mutagenesis protocol (Zheng *et al.*, 2004), two complementary primers were designed: 5'-ACCTTGACCAGCCC-TATGTTTCATCGGCTACTCG-3' and 5'-CGAGTAGCCGATGAA-CATAG-GGCTGGTCAAGGT-3'. A mutation site was created in the primers that replaced leucine with methionine (Fig. 1). The PCR program for site-directed mutagenesis was as follows: initial denaturation for 6 min at 367 K, 25 cycles of 367 K for 40 s, 323 K for 40 s and 341 K for 6 min, and a final extension for 10 min at 341 K. The PCR products were purified and then treated with the *DpnI* restriction enzyme (Fermentas) to eliminate the parental methylated DNA from the newly synthesized and unmethylated mutant DNA.

2.2. Expression and purification

A recombinant expression vector containing the native *mohrip2* gene was transformed into *Escherichia coli* Codon Plus competent cells. The cells were cultured in LB medium at 310 K to an OD₆₀₀ of 0.6 and then induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 310 K.

The cells were harvested by centrifugation for 15 min at 5000 rev min⁻¹. The sedimented cells were resuspended in Ni-IMAC (immobilized metal-affinity chromatography) matrix binding buffer (50 mM Tris, 200 mM NaCl, pH 8.5) and disrupted by sonication. Because the protein was expressed in inclusion bodies, after centrifugation for 40 min at 18 000g the supernatant was discarded and the precipitate was processed in the following steps: the precipitate was resuspended in buffer A (50 mM Tris, 5 mM EDTA pH 8.5) followed by buffer B (buffer A containing 2% Triton X-100), disrupted by sonication, and the supernatant was then discarded after centrifugation for 40 min at 18 000g. The precipitate was sequentially washed again using ddH₂O, buffer B and buffer A, and the supernatant was discarded after centrifugation for 40 min at 18 000g. The precipitate was resuspended in the appropriate denaturing buffer (100 mM Tris, 2 M urea pH 12) and placed at room temperature for 30–60 min until the precipitate was completely dissolved. The solution was then

Figure 1

The position of the Leu-Met mutant in the target protein, and the TEV cleavage site and His-tag sequence in the pET-30 TEV/LIC vector.

Figure 2
SDS-PAGE of the target protein used for crystallization. The left lane contains molecular-mass markers (labelled in kDa).

clarified by centrifugation for 40 min at 18 000g. The supernatant was carefully transferred to a clean container. Finally, according to the dilution method for renaturation *in vitro* (Fang & Huang, 2001), the supernatant was added dropwise into refolding buffer (50 mM Tris, 2 M urea, 5% sucrose pH 8.5) in 1:10(v/v) proportions and incubated at 277 K for 12 h (Singh *et al.*, 2008).

The renaturation solution was loaded onto an Ni-IMAC matrix column and washed with washing buffer (binding buffer plus 20 mM imidazole), and the protein was then eluted using elution buffer (binding buffer plus 500 mM imidazole). For further purification, the eluted protein was concentrated and diluted with a low-salt buffer (50 mM Tris pH 8.5) and then incubated with approximately 0.4 mg TEV protease (purified in our laboratory) for 12 h at 289 K to remove the 6×His tag from the Q/S cleavage site [three extra amino-acid residues (S, N, A) remained on the target protein from the vector] (Fig. 1). Ion-exchange chromatography was then implemented with a linear NaCl elution gradient from 0 to 1 M with a

HiTrap Q HP column (GE Healthcare). The eluted peak was concentrated and loaded onto a HiLoad 16/60 Superdex 200 size-exclusion column (GE Healthcare) in binding buffer for further refining. Recombinant MoHrip2 was detected by SDS-PAGE (Fig. 2) and concentrated to the appropriate concentration for further use.

The recombinant expression vector containing the *mohrip2-L78M* gene was transformed into *E. coli* B834 competent cells, which are a methionine-auxotrophic strain. The cells were cultured in 1 l medium A with 50 µg ml⁻¹ methionine at 310 K to an OD₆₀₀ of 1.0 and the cells were collected by centrifugation for 15 min at 5000 rev min⁻¹. The pellet was resuspended in 1 l medium A (without methionine) and cultured for 8 h at 310 K. Then, the fermentation broth was supplemented with selenomethionine to 50 µg ml⁻¹ and cultured for an additional 30 min. A final concentration of 0.2 mM IPTG was added and the fermentation broth was cultured for 6 h at 310 K to induce target protein expression. The target protein containing the Se atom was purified as stated above.

2.3. Crystallization

The Index, Crystal Screen, Crystal Screen 2, PEG/Ion and PEG/Ion 2 screen kits from Hampton Research were used for initial screening using the sitting-drop vapour-diffusion method at 289 K. 1 µl protein solution containing 2 mg ml⁻¹ target protein was mixed with 1 µl reservoir solution in the drop and equilibrated against 100 µl reservoir solution. Approximately 1 week later, the crystals appeared in three conditions: Index condition Nos. 5 and 6, and Crystal Screen condition No. 4. All of these conditions contained 2.0 M ammonium sulfate as the precipitant with different buffer pH values. The crystallization conditions were further optimized by slightly modifying the buffer and the concentrations of the precipitant and target protein. Finally, single crystals were obtained with an optimized precipitant concentration of 2.2 M ammonium sulfate in Tris buffer pH 8.0 containing 2 mg ml⁻¹ protein. These optimized crystals grew to at least 200 × 100 × 50 µm within 1 month (Fig. 3a) and yielded a good diffraction pattern (Fig. 4a).

Selenomethionine-labelled MoHrip2-L78M was crystallized under the same conditions and the crystals (Fig. 3b) yielded a good diffraction pattern (Fig. 4b).

Figure 3
Native MoHrip2 (a) and selenomethionine-labelled MoHrip2-L78M (b) crystals grown in 2.2 M ammonium sulfate and Tris buffer pH 8.0.

Table 1

Data-collection and processing statistics.

Values in parentheses are for the outer resolution shell.

Data set	Native	Selenium derivative
X-ray source	Rigaku MicroMax-007 HF	1W2B, BSRF
Wavelength (Å)	1.5418	0.9792
Detector	R-Axis HTC image plate	MAR 345 image plate
Crystal parameters		
Space group	$P2_1$	$P2_1$
Unit-cell parameters		
a (Å)	36.24	36.78
b (Å)	43.51	43.76
c (Å)	74.01	73.92
β (°)	92.88	92.84
Resolution (Å)	2.0 (2.18–2.00)	1.8 (1.86–1.80)
Mosaicity	0.54	0.39
Solvent content (%)	37	37
Molecules per asymmetric unit	2	2
Data processing		
No. of observed reflections	81303 (5034)	93203 (6670)
No. of unique reflections	19830 (1678)	21184 (1906)
Completeness (%)	99.2 (94.4)	96.6 (86.4)
Multiplicity	4.1 (3.0)	4.4 (3.5)
$R_{\text{merge}}^{\dagger}$ (%)	6.4 (36.4)	5.7 (28.8)
Average $I/\sigma(I)$	13.3 (1.6)	16.8 (2.3)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of i observations for symmetry-related reflections of hkl .

2.4. Data collection and processing

To avoid ice rings, the crystals were soaked for several seconds in reservoir solution containing 20% glycerol and then mounted in the beamline with a nylon loop and flash-cooled in a nitrogen-gas stream at 100 K. The native data set was collected at 1.5418 Å using an R-Axis HTC image plate with an in-house Rigaku MicroMax-007 HF X-ray source at Nankai University, Tianjin, People's Republic of China. The crystal-to-detector distance was 160 mm and 180 frames were collected with 1° oscillation at 2.0 Å resolution. The seleno-

methionine-labelled crystals were mounted on the BSRF (Beijing Synchrotron Radiation Facility) 1W2B beamline at the Institute of High Energy Physics, Chinese Academy of Sciences, Beijing, People's Republic of China. In addition, a SAD (single-wavelength anomalous dispersion) data set was collected at 0.9792 Å using a MAR 345 image plate (MAR Research). The crystal-to-detector distance was 160 mm and 270 frames were collected with 1° oscillation at 1.8 Å resolution.

Both of the data sets were indexed, integrated and scaled with the *HKL-2000* package (Otwinowski & Minor, 1997). The crystals belonged to the $P2_1$ space group with two molecules per asymmetric unit. The data-collection statistics are shown in Table 1. Structure determination and associated functional experiments are in progress.

3. Discussion

In this study, we expressed and crystallized the protein effector MoHrip2 and its selenomethionine derivative. The crystals diffracted strongly and had a Matthews coefficient (Matthews, 1968) of 1.96 Å³ Da^{−1} (solvent content of 37%). The SAD signal for selenium was sufficiently strong to determine phase information for the crystals.

Because there was no known homologous structure, molecular replacement (MR) could not be used to determine the initial phase of MoHrip2. We chose the SAD method with selenium-derivative crystals to solve the phase problem. To ensure a sufficient anomalous signal, we introduced an additional methionine by site-directed mutagenesis based on predictions of the MoHrip2 secondary structure.

Our laboratory has isolated several effector proteins from plant pathogenic fungi, including Hrip1, PevD1, MoHrip1 (Kulye *et al.*, 2012; Wang *et al.*, 2012; Chen *et al.*, 2012) and MoHrip2, and all of these proteins have the same effector function and could induce HR. Although these proteins are from different pathogens whose hosts

Figure 4

The diffraction pattern of native MoHrip2 (a) and selenomethionine-labelled MoHrip2-L78M (b).

are different, some have sequence homology (PevD1 has 47% sequence homology with MoHrip1) or similar structures. Therefore, we presume that different fungi effectors have similar mechanisms or signalling pathways for inducing plant innate immunity. Although PevD1 was isolated from *Verticillium dahliae*, whose host belongs to the dicotyledons, and *M. oryzae*, which belongs to the monocotyledons, our other experiments demonstrated that PevD1 and MoHrip2 can induce HR in tobacco and *Arabidopsis thaliana*. However, the detailed mechanism of these effectors in inducing plant innate immunity is unclear. We should identify the corresponding receptors or target proteins in plant cells for the effectors and further investigate the downstream response. Finally, we can determine the complete signalling pathway that effectors perform.

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