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Purification, crystallization and preliminary X-ray diffraction analysis of the effector protein MoHrip1 from *Magnaporthe oryzae*

The effector protein MoHrip1 from the pathogenic fungus *Magnaporthe oryzae* was purified and crystallized using the sitting-drop vapour-diffusion method. Native crystals appeared in a solution composed of 0.005 M cobalt(II) chloride hexahydrate, 0.005 M nickel(II) chloride hexahydrate, 0.005 M cadmium chloride hydrate, 0.005 M magnesium chloride hexahydrate, 0.1 M HEPES pH 7.5, 12% (w/v) polyethylene glycol 3350. A native data set was collected to 1.9 Å resolution at 100 K using an in-house X-ray source. The structure of MoHrip1 was successfully determined by molecular replacement using a homologous structure.

1. Introduction

In nature, plants are exposed to a broad range of potential pathogens during their life cycle, including bacteria, oomycetes and fungi. These phytopathogens have different life cycles and infection strategies. However, plants have also established a complicated immune defence system during co-evolution with pathogens. During plant–pathogen interactions, plant pathogens secrete effectors to alter host-cell structure and function for modulating plant defence circuitry and enabling parasitic colonization (Hogenhout *et al.*, 2009). These alterations either facilitate pathogenic infection or trigger plant defence responses or both (Hogenhout *et al.*, 2009). *Magnaporthe oryzae*, the causal agent of rice blast, is a plant-pathogenic fungus that causes losses of rice yields between 10 and 30% (Ebbole, 2007; Talbot, 2003). To date, many effectors have been identified from *M. oryzae*, including proteins and glycoproteins (Chen *et al.*, 2012; Kanoh *et al.*, 1993; Liu *et al.*, 2010; Schafrath *et al.*, 1995).

The three-dimensional structure of some proteins plays a critical role in determining their biological function and mechanism of action. In recent years, the three-dimensional structures of some effectors, such as β -cryptogin (CRY; Boissy *et al.*, 1996), Ptr ToxA (a proteinaceous toxin produced by *Pyrenophora tritici-repentis*; Sarma *et al.*, 2005), necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs; Ottmann *et al.*, 2009), have been obtained. In particular, some complexes of the effector and receptor have been acquired, including the crystal structure of AtCERK1-ECD [lysine motif (LysM)-containing ectodomain of cell surface receptor chitin elicitor receptor kinase 1 of *Arabidopsis* (AtCERK1)] complexed with chitin (Liu *et al.*, 2012), which provided new insights into the molecular basis of pathogen–plant interactions. Therefore, elucidation of effector protein structures is of great interest.

We have previously identified a new effector from *M. oryzae* which can trigger a defence response in rice, and designated this protein *M. oryzae* hypersensitive response-inducing protein 1 (MoHrip1; Chen *et al.*, 2012). Here, we report the expression, purification and crystallization of MoHrip1 protein and the subsequent sample optimizations that led to high-diffraction-quality crystals. A set of high-resolution native data was successfully collected using an in-house X-ray source. Based on the structure of PevD1 (a protein elicitor from *Verticillium dahliae*) previously obtained in our laboratory (Han *et al.*, 2012), the molecular-replacement (MR) method was performed to solve the phase problem. Finally, the structure of MoHrip1 was successfully determined.

2. Materials and methods

2.1. Cloning

The full-length *mohrip1* gene (GenBank accession No. JQ231215.1) consists of 429 bp, encoding 142 amino-acid residues with a theoretical molecular weight of 14.322 kDa. Analysis of the deduced amino-acid sequence using the *SignalP* 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) revealed that it contains a 16 amino-acid signal peptide, indicating that MoHrip1 is a secreted protein, which is consistent with the observation that it was isolated from the culture filtrate of *M. oryzae*. After amplifying with the

forward primer 5'-TACTTCCAATCCAATGCCGCCCCCTGCC-CCGCAGGCG-3' and the reverse primer 5'-TTATCCAATTC-CAATGCTAAGCGGAGCCGTCATGGC-3', the sequence without the signal peptide, encoding amino acids 17–142, was cloned into the pET30-TEV/LIC vector (Novagen, USA) using ligation-independent cloning as described by Aslanidis & Jong (1990). The PCR program was as follows: 367 K for 5 min, followed by 30 cycles of 367 K for 30 s, 331 K for 30 s and 345 K for 30 s, and a final extension at 345 K for 10 min. Finally, the clone of the *mohrip1* gene insertion was verified by DNA sequencing.

2.2. Expression and purification

The recombinant expression vector consisting of *mohrip1* gene insertion was transformed into *Escherichia coli* Codon Plus competent cells to express the effector as a C-terminally 6×His-tagged protein. The bacteria were first grown in LB medium at 310 K to an OD₆₀₀ of 0.8 and expression was then induced by the addition of 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at 289 K. 16 h after induction, the cells were collected by centrifugation and resuspended in Ni-IMAC (IMAC = immobilized metal-affinity chromatography) matrix buffer A (25 mM Tris, 500 mM NaCl pH 8.0). After ultrasonication and centrifugation, the supernatant was loaded onto an Ni-IMAC matrix-affinity chromatography column (GE Healthcare, USA), followed by washing with buffer B (buffer A plus 20 mM imidazole) and eluted with buffer C (buffer A plus 200 mM imidazole). The eluted protein was then concentrated by ultrafiltration, buffer C was replaced by the low-salt buffer D (25 mM Tris pH 8.0) and the protein was digested with TEV protease at 289 K for 8 h to remove the N-terminal His tag. The further purification of MoHrip1 consisted of two sequential procedures as follows: ion-exchange chromatography performed using a linear NaCl elution gradient from 0 to 1 M with a HiTrap Q column (GE Healthcare, USA) and size-exclusion chromatography performed in buffer E (25 mM Tris, 200 mM NaCl pH 8.0) with a HiLoad 16/60 Superdex-200 size-exclusion column (GE Healthcare, USA). The purified protein was detected by SDS-PAGE and concentrated for further use.

2.3. Crystallization

Hampton Research screen kits (Index, Crystal Screen, Crystal Screen 2, PEG/Ion and PEG/Ion 2) and the sitting-drop vapour-diffusion method were selected for the initial screening. 1 µl protein solution at a series of concentrations from 2 to 8 mg ml⁻¹ was manually mixed with 1 µl reservoir solution in the drop and equilibrated against 100 µl reservoir solution. After approximately 1 month, three types of crystals in three different conditions were obtained during the original screening, but only those that appeared in Index condition No. 64 [0.005 M cobalt(II) chloride hexahydrate, 0.005 M nickel(II) chloride hexahydrate, 0.005 M cadmium chloride hydrate, 0.005 M magnesium chloride hexahydrate, 0.1 M HEPES pH 7.5, 12% (w/v) polyethylene glycol 3350] had good diffraction quality. The crystallization condition was further optimized by slightly modifying the pH and the concentrations of both the precipitant and the protein. These changes had little impact on crystallization; the pH was modified in the range from 7.0 to 8.0, the precipitant concentration from 10 to 15% (w/v) and the protein concentration from 2 to 4 mg ml⁻¹. Under the modified condition, the crystals grew to sizes in excess of 100 × 100 × 100 µm and yielded a good diffraction pattern (Fig. 1). Moreover, among the metal ions in the initial crystallization trial, cadmium was essential for crystallization, which was consistent with the experimental observation that no crystals appeared in

Figure 1

(a) Crystals of MoHrip1 grew in 0.005 M cobalt(II) chloride hexahydrate, 0.005 M nickel(II) chloride hexahydrate, 0.005 M cadmium chloride hydrate, 0.005 M magnesium chloride hexahydrate, 0.1 M HEPES pH 7.5, 12% (w/v) polyethylene glycol 3350. (b) An X-ray diffraction image from a MoHrip1 crystal. The edge of the detector corresponds to a resolution of 2.00 Å and is represented as a circle. The crystal-to-detector distance was 160 mm and the exposure time for data collection was 2 min per image.

Table 1

Data-collection and processing statistics.

Values in parentheses are for the outer resolution shell.

X-ray source	Rigaku MicroMax-007 HF
Wavelength (Å)	1.5418
Detector	R-Axis HTC image plate
Space group	<i>P</i> 2 ₁ 3
Unit-cell parameters	
<i>a</i> (Å)	78.35
<i>b</i> (Å)	78.35
<i>c</i> (Å)	78.35
α (°)	90
β (°)	90
γ (°)	90
Resolution (Å)	50–1.93 (2.00–1.93)
Mosaicity (°)	0.43
Molecules per asymmetric unit	1
No. of observed reflections	264854 (26432)
No. of unique reflections	12370 (1235)
Completeness (%)	100 (99.8)
Multiplicity	21.8 (20.9)
<i>R</i> _{merge} † (%)	5.2 (14.9)
Average <i>I</i> / σ (<i>I</i>)	18.6 (2.7)

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

crystallization conditions without cadmium and the experimental data derived from data processing.

2.4. Data collection and processing

Data collection was performed at a wavelength of 1.54 Å (Cu *K*α) using an in-house Rigaku MicroMax-007 HF X-ray source with an R-Axis HTC image plate. Ahead of data collection, the crystal was soaked for several seconds in cryoprotectant composed of reservoir solution supplemented with 20% (v/v) glycerol. The crystal was then mounted on the beamline with a nylon loop; the crystal-to-detector distance was 160 mm. The crystal was cooled in a nitrogen-gas stream at 100 K throughout the process of data collection. The oscillation angle was 1.0° and the exposure time for data collection was 2 min per image; 180 images were collected.

The data set was indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). The crystals belonged to space group *P*2₁3, with one monomer per asymmetric unit. As calculated from the Matthews coefficient (Matthews, 1968), the solvent content is approximately 57%. Moreover, we found that there were cadmium ions in the electron-density maps calculated from the protein crystals, which is consistent with the experimental observation that cadmium ions were essential for crystallization. Statistics of data collection and processing are shown in Table 1. Previously we have solved the structure of the effector protein PevD1. Owing to the 42% sequence homology between the two effectors, we tried to use the MR method in *PHENIX* (Adams *et al.*, 2010) to solve the phase problem and to determine the structure of MoHrip1 based on the high-resolution native data set. The structure determination and associated functional experiment of MoHrip1 are in progress.

3. Discussion

In this study, we expressed, purified and crystallized MoHrip1, an effector from *M. oryzae*. The crystals displayed very good diffraction quality and had a Matthews coefficient of 2.87 Å³ Da^{−1}, corresponding to a solvent content of 57%. As a homologous structure,

PevD1, was available in our laboratory, we performed the MR method to solve the phase problem and succeeded in determining the structure of MoHrip1. An interpretable electron-density map was then obtained and further refinement is under way.

Both PevD1 and MoHrip1 have typical characteristics of elicitors and can induce hypersensitive responses in tobacco (Chen *et al.*, 2012; Wang *et al.*, 2012). PevD1 is from *V. dahliae*, the hosts of which are dicotyledonous plants such as cotton or *Arabidopsis*, while MoHrip1 is derived from rice blast fungus, the hosts of which are monocotyledonous rice plants. Although monocotyledons and dicotyledons are apparently different in evolution, the proteins from *V. dahliae* and *M. oryzae* still retain a conserved structure during long-term plant–pathogen interaction. Therefore, we have reason to presume that fungi infesting different hosts are likely to retain a similar interaction during the evolutionary process; in other words, proteins with similar structures may function in a similar manner in the process of effector–receptor recognition during infection. In reverse, we also have reason to believe that the defence systems of different classes of plants may be likely to retain some key and conservative defence mechanisms during long-term evolution. Consistent with this presumption, the results of our studies showed that host plants treated with MoHrip1 or PevD1 exhibited enhanced systemic acquired resistance to their pathogens, respectively.

Based on our current study, the molecular mechanism of both effectors in the development of host-plant resistance against pathogens should be further investigated. We should determine the targets of these proteins in host cells and their functions in the host response to pathogen infection. Finally, this would allow us to establish a model to elaborate the molecular mechanism underlying these functions.

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References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Aslanidis, C. & de Jong, P. J. (1990). *Nucleic Acids Res.* **18**, 6069–6074.
- Boissy, G., de La Fortelle, E., Kahn, R., Huet, J. C., Bricogne, G., Pernollet, J. C. & Brunie, S. (1996). *Structure*, **4**, 1429–1439.
- Chen, M., Zeng, H., Qiu, D., Guo, L., Yang, X., Shi, H., Zhou, T. & Zhao, J. (2012). *PLoS One*, **7**, e37654.
- Ebbold, D. J. (2007). *Annu. Rev. Phytopathol.* **45**, 437–456.
- Han, L., Liu, Z., Liu, X. & Qiu, D. (2012). *Acta Cryst.* **F68**, 802–805.
- Hogenhout, S. A., Van der Hoorn, R. A. L., Terauchi, R. & Kamoun, S. (2009). *Mol. Plant Microbe Interact.* **22**, 115–122.
- Kanoh, H., Haga, M. & Sekizawa, Y. (1993). *J. Pestic. Sci.* **18**, 325–332.
- Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., Zhou, J. M. & Chai, J. (2012). *Science*, **336**, 1160–1164.
- Liu, J., Wang, X., Mitchell, T., Hu, Y., Liu, X., Dai, L. & Wang, G. L. (2010). *Mol. Plant Pathol.* **11**, 419–427.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Ottmann, C., Luberacki, B., Küfner, I., Koch, W., Brunner, F., Weyand, M., Mattinen, L., Pirhonen, M., Anderlüh, G., Seitz, H. U., Nürnberger, T. & Oecking, C. (2009). *Proc. Natl Acad. Sci. USA*, **106**, 10359–10364.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Sarma, G. N., Manning, V. A., Ciuffetti, L. M. & Karplus, P. A. (2005). *Plant Cell*, **17**, 3190–3202.
- Schaffrath, U., Scheinpflug, H. & Reisener, H. (1995). *Physiol. Mol. Plant Pathol.* **46**, 293–307.
- Talbot, N. J. (2003). *Annu. Rev. Microbiol.* **57**, 177–202.
- Wang, B., Yang, X., Zeng, H., Liu, H., Zhou, T., Tan, B., Yuan, J., Guo, L. & Qiu, D. (2012). *Appl. Microbiol. Biotechnol.* **93**, 191–201.