Expression, purification, crystallization and preliminary X-ray diffraction analysis of the effector-interaction domain of the resistance protein RGA5-A from *Oryza sativa* L. *japonica*

Dan Huang, Yanan Zhang, Yanxiang Zhao, Junfeng Liu* and You-Liang Peng

Received 11 October 2014

Accepted 24 December 2014

China Agricultural University, 2 Yuanmingyuan Xilu, Beijing 100193, People's Republic of China. *Correspondence e-mail: jliu@cau.edu.cn

Keywords: *Oryza sativa* L. *japonica*; resistance protein; RGA5-A.

RGA5-A, a component of the Pia resistance-protein complex (RGA4/RGA5-A) from *Oryza sativa* L. *japonica*, has the ability to interact physically with the effector protein AVR-Pia from *Magnaporthe oryzae* via its effector-interaction domain RGA5-A_S. The interaction between RGA5-A and AVR-Pia relieves the repression of RGA4, leading to AVR-independent cell death by the freed RGA4. To further understand the details of this interaction, the effector-interaction domain RGA5-A_S was expressed in *Escherichia coli* and purified to homogeneity. The purified recombinant protein His-RGA5-A_S was successfully crystallized using the sitting-drop vapour-diffusion method. A single crystal obtained using 0.2 M ammonium citrate, 25% (w/v) PEG 3350 diffracted to 2.43 Å resolution. It belonged to space group $P4_122$ or $P4_322$, with unit-cell parameters $a = b = 55.2$, $c = 78.2$ Å, $\alpha = \beta = \gamma = 90^\circ$.

1. Introduction

The rice blast disease caused by *Magnaporthe oryzae* is one of the most serious fungal diseases worldwide. The specific interaction between avirulence genes in *M. oryzae* and resistance genes in rice can be well explained by the gene-for-gene system. The gene-for-gene hypothesis proposes that defence responses are activated by recognition between a host resistance gene and a corresponding pathogen avirulence gene (Flor, 1971). 23 rice resistance genes to rice blast fungus have been cloned, while only five corresponding avirulence genes, AvrPita, AvrPik, AvrPiz-t, AvrPia and Avr1-CO39, have been identified (Pieterse *et al.*, 2014). Among these resistance proteins, Pita and Pia have been confirmed to directly interact with AvrPita and AvrPia, respectively (Jia *et al.*, 2000). Pia, as a resistance allele, includes two CC-NB-LRR-coding genes: RGA4 and RGA5 (Okuyama *et al.*, 2011). The two genes are both required for the recognition and the defence response of the *M. oryzae* effector AVR-Pia. Further study showed that RGA5 directly binds to RGA4 to repress an RGA4-triggered effector-independent cell-death response (Césari *et al.*, 2014). RGA5 seems to play a central role in the recognition because of its direct interaction with both AVR-Pia and RGA4. RGA5-A, the active and functional transcript of RGA5, has an AVR receptor-binding domain RGA5-A_S (RGA5-A₉₈₂₋₁₁₁₆; Cesari *et al.*, 2013). However, owing to limited biochemical and biophysical studies, the molecular mechanism by which RGA5 recognizes AVR-Pia remains unclear.

© 2015 International Union of Crystallography

Table 1

Macromolecule-production information.

Source organism	<i>O. sativa</i> L. <i>japonica</i>
DNA source	Chemically synthesized fragment
Expression vector	pHAT2
Expression host	<i>E. coli</i> strain BL21 (DE3)
Complete amino-acid sequence of the construct produced†	MSHHHHHSMDSLNMESVVESALTGQRTKIVVKV-HMPCGKSRAMALAASVNGVDSVEITGEDKDLVVVGRGIDPVRLVALLREKCGLAELLMVEL-VEKEKTQLAGGKKGAYKKHPTYNLSPFDYVEY-PPSAPIMQDINPCSTM

Here, we report the expression, purification and crystallization of RGA5-A_S and the collection of 2.43 Å resolution X-ray diffraction data from crystals of recombinant RGA5-A_S. Further structural characterization of RGA5-A_S will provide new insight into the mechanism of its interaction with the effector Avr-Pia.

2. Materials and methods

2.1. Expression and purification of recombinant RGA5-A_S

The amino-acid sequence of RGA5-A_S (GenBank BAK39930.1) is shown in Table 1. The DNA fragment encoding RGA5-A_S was chemically synthesized and sub-cloned into pHAT2 vector using NcoI and XhoI restriction sites to produce an expression vector encoding RGA5-A_S with an N-terminal His₆ tag by TaiHe Biotechnology Co. Ltd. The vector was transformed into *Escherichia coli* BL21 (DE3) cells. Transformants were cultured in LB medium containing 100 µg ml⁻¹ ampicillin until the OD₆₀₀ reached about 0.8. Expression of the recombinant protein was induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 291 K. Cells were harvested by centrifugation at 5000g for 10 min at 277 K.

Figure 1
15% SDS-PAGE analysis of the purified recombinant His-RGA5-A_S protein. The left lane contains molecular-weight marker (labelled in kDa).

The harvested cells were resuspended in buffer A (50 mM NaH₂PO₄/Na₂HPO₄ pH 7.0, 500 mM NaCl, 1 mM DTT) with 20 mM imidazole and were disrupted by ultrasonication. After centrifugation at 12 000g for 30 min, the supernatant was loaded onto a 3 ml Ni-NTA agarose column that had been pre-equilibrated with buffer A. The column was first washed with ten column volumes of buffer A. Five column volumes of buffer A with different imidazole concentrations were then used to elute the bound protein. The recombinant RGA5-A_S mainly eluted in buffer A with 200 mM imidazole. The pooled fraction containing the protein of interest was concentrated and then loaded onto a Superdex 200 10/300 GL gel-filtration chromatography column (GE Healthcare) pre-equilibrated with buffer A at a flow rate of 0.5 ml min⁻¹. The target protein was collected and concentrated to about 5 mg ml⁻¹ with an Amicon Ultra-15 centrifugal filter with 10 kDa molecular-weight cutoff (Millipore) (Fig. 1). All steps were carried out at 277 K.

2.2. Crystallization

Crystallization was performed at 291 K using the sitting-drop vapour-diffusion method with an Oryx4 robot (Douglas Instruments Ltd; <http://www.douglas.co.uk>). A 0.5 µl mixture of the concentrated protein solution described above and reservoir solution in different proportions was placed on 2-well MRC plates (Hampton Research) and equilibrated against 70 µl reservoir solution. The initial crystallization

Figure 2
Crystals of RGA5-A_S. (a) Initial crystals obtained from 0.2 M ammonium citrate, 25% (w/v) PEG 3350. (b) Optimized crystals obtained from 0.2 M ammonium citrate, 25% (w/v) PEG 3350 by seeding with the initial crystals.

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	2-well MRC plates
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	5–6
Buffer composition of protein solution	50 mM NaH ₂ PO ₄ /Na ₂ HPO ₄ pH 7.0, 500 mM NaCl, 1 mM DTT
Composition of reservoir solution	0.2 M ammonium citrate, 25% (w/v) PEG 3350
Volume and ratio of drop	0.5 µl, 2:2:1 protein solution:reservoir solution:seed stock
Volume of reservoir (µl)	70

screening was performed using commercial crystal screening kits such as PEG/Ion from Hampton Research (<http://www.hamptonresearch.com>) and MSCG-1, MSCG-2 and MSCG-3 from Microlytic (<http://www.microlytic.com>). Mini-crystals were observed from several screening conditions within two weeks (Fig. 2*a*). To improve the quality of the crystals, seeding experiments were performed. A seed stock was prepared according to the manual from Hampton Research. 0.2 µl protein solution mixed with 0.2 µl reservoir solution and 0.1 µl seed stock was then equilibrated against 70 µl reservoir solution (Table 2).

2.3. Data collection and processing

The crystals were soaked in reservoir solution supplemented with 20% (v/v) glycerol for about 2 min and then flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K on beamline BL-17U1 at Shanghai Synchrotron Research Facility (SSRF). The data were indexed and scaled with *xia2* in *CCP4* (Winter, 2010; Winn *et al.*, 2011). Data-collection and processing statistics are shown in Table 3.

Figure 3
X-ray diffraction image of an RGA5-A_S crystal.

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL-17U1, SSRF
Wavelength (Å)	0.9792
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	<i>P</i> ₄ 22 or <i>P</i> ₄ 32
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 55.2, <i>c</i> = 78.2, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.366
Resolution range (Å)	55.19–2.43
Total No. of reflections	65299 (7051)
No. of unique reflections	4956 (514)
Completeness (%)	100 (100)
Multiplicity	13.2 (13.7)
$\langle I/\sigma(I) \rangle$	16.8 (4.8)
<i>R</i> _{int} (%)	10.7 (60.4)
Overall <i>B</i> factor from Wilson plot (Å ²)	41.68

3. Results and discussion

Recombinant His-RGA5-A_S protein was efficiently expressed in *E. coli* BL21 (DE3) cells. This protein was purified to high homogeneity by Ni-agarose affinity chromatography followed by gel-filtration chromatography. 10 mg of recombinant His-RGA5-A_S could be purified from a 1 l cell culture. The migration of the protein on 15% SDS-PAGE showed an estimated molecular mass of 15–16 kDa (Fig. 1), which is close to the calculated value based on the amino-acid sequence.

Initial small crystals were observed from several screening conditions containing ammonium citrate within two weeks (Fig. 2*a*). Seeding was used as an effective rescue strategy to improve the crystal quality. After optimization, the crystal with the highest quality was obtained using 0.2 M ammonium citrate, 25% (w/v) PEG 3350 within one week (Fig. 2*b*).

X-ray diffraction data were collected to a resolution of 2.43 Å (Fig. 3). The crystal belonged to space group *P*₄22 or *P*₄32, with unit-cell parameters *a* = *b* = 55.2, *c* = 78.2 Å, $\alpha = \beta = \gamma = 90^\circ$. Table 3 shows the statistics of the data collected at SSRF. The Matthews coefficient of 2.32 Å³ Da⁻¹ suggests the presence of one molecule per asymmetric unit, with a solvent content of 47.0% (Matthews, 1968). Molecular replacement using homology models to obtain the phase is being undertaken and we are also working on obtaining a solution using the MAD or SAD method.

Acknowledgements

We thank the staff of Shanghai Synchrotron Research Facility (SSRF) beamline BL-17U1 and Beijing Synchrotron Research Facility (BSRF) stations 3W1A and 1W2B for help with crystal screening and data collection. This research was supported by the 973 Project (No. 2012CB114000) of the Ministry of Science and Technology (MOST), People's Republic of China and the PCSIRT Project (IRT1042) and the 111 Project (B13006) and the NCET Project (NCET-12-0518) of the Ministry of Education, People's Republic of China.

Thanks are also given to Dr Xiong Xiaoli (Alex) at MRC–NIMR, London, England for his critical reading of the manuscript.

References

- Césari, S., Kanzaki, H., Fujiwara, T., Bernoux, M., Chalvon, V., Kawano, Y., Shimamoto, K., Dodds, P., Terauchi, R. & Kroj, T. (2014). *EMBO J.* **33**, 1941–1959.
- Cesari, S., Thilliez, G., Ribot, C., Chalvon, V., Michel, C., Jauneau, A., Rivas, S., Alaux, L., Kanzaki, H. & Okuyama, Y. (2013). *Plant Cell*, **25**, 1463–1481.
- Flor, H. H. (1971). *Annu. Rev. Phytopathol.* **9**, 275–296.
- Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P. & Valent, B. (2000). *EMBO J.* **19**, 4004–4014.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Okuyama, Y., Kanzaki, H., Abe, A., Yoshida, K., Tamiru, M., Saitoh, H., Fujibe, T., Matsumura, H., Shenton, M., Galam, D. C., Undan, J., Ito, A., Sone, T. & Terauchi, R. (2011). *Plant J.* **66**, 467–479.
- Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. & Bakker, P. A. (2014). *Annu. Rev. Phytopathol.* **52**, 347–375.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Winter, G. (2010). *J. Appl. Cryst.* **43**, 186–190.