Crystallization of the rice immune receptor RGA5A_S with the rice blast fungus effector AVR1-CO39 prepared *via* mixture and tandem strategies

Liwei Guo,^a Yikun Zhang,^a Mengqi Ma,^a Qiang Liu,^a Yanan Zhang,^a
Youliang Peng^{a,b} and Junfeng Liu^{a*}

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^aKey Laboratory of Pest Monitoring and Green Management, MOA and College of Plant Protection, China Agricultural University, No. 2 Yunanmingyuanxilu, Beijing 100193, People's Republic of China, and ^bState Key Laboratory of Agrobiotechnology, China Agricultural University, No. 2 Yunanmingyuanxilu, Beijing 100193, People's Republic of China. *Correspondence e-mail: jliu@cau.edu.cn

RGA5 is a component of the Pia resistance-protein pair (RGA4/RGA5) from *Oryza sativa* L. *japonica*. It acts as an immune receptor that directly recognizes the effector AVR1-CO39 from *Magnaporthe oryzae* *via* a C-terminal non-LRR domain (RGA5A_S). The interaction between RGA5A_S and AVR1-CO39 relieves the repression of RGA4, leading to effector-independent cell death. To determine the structure of the complex of RGA5A_S and AVR1-CO39 and to understand the details of this interaction, the complex was prepared by fusing the proteins together, by mixing them *in vitro* or by co-expressing them in one host cell. Samples purified *via* the first two strategies were crystallized under two different conditions. A mixture of AVR1-CO39 and RGA5A_S (complex I) crystallized in 1.1 M ammonium tartrate dibasic, 0.1 M sodium acetate–HCl pH 4.6, while crystals of the fusion complex RGA5A_S-TEV-AVR1-CO39 (complex II) were grown in 2 M NaCl. The crystal of complex I belonged to space group $P3_121$, with unit-cell parameters $a = b = 66.2$, $c = 108.8$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The crystals diffracted to a Bragg spacing of 2.4 Å, and one molecule each of RGA5A_S and AVR1-CO39 were present in the asymmetric unit of the initial model. The crystal of complex II belonged to space group $I4$, with unit-cell parameters $a = b = 137.4$, $c = 66.2$ Å, $\alpha = \beta = \gamma = 90^\circ$. The crystals diffracted to a Bragg spacing of 2.72 Å, and there were two molecules of RGA5A_S and two molecules of AVR1-CO39 in the asymmetric unit of the initial model. Further structural characterization of the interaction between RGA5A_S and AVR1-CO39 will lead to a better understanding of the mechanism underlying effector recognition by R proteins.

1. Introduction

Rice is an important staple food for more than 50% of the population of the world. However, the rice plant is constantly affected by biotic (Robert-Seilaniantz *et al.*, 2011) and abiotic (Hua, 2013) stresses from the seedling to the adult stages. Rice blast (Ribot *et al.*, 2008), which is caused by the ascomycete fungal pathogen *Magnaporthe oryzae*, is the most devastating disease of rice and represents a major threat to food security. Specific interactions between the avirulence (*Avr*) genes in *M. oryzae* and the matching resistance (*R*) genes in rice induce strong defences associated with a hypersensitive response (HR; Dodds & Rathjen, 2010).

To date, more than 100 major rice-blast *R* genes have been genetically characterized (Sharma *et al.*, 2012). Among 25 cloned *R* genes, 23 encoded cytoplasmic nucleotide-binding

Table 1
Macromolecule-production information.

Protein	RGA5A_S	AVR1-CO39-6×His	6×His-RGA5A_S-TEV-AVR1-CO39
Source organism	<i>O. sativa</i> L. <i>japonica</i>	<i>M. oryzae</i>	<i>O. sativa</i> L. <i>japonica</i> and <i>M. oryzae</i>
DNA source	Chemically synthesized fragment	Chemically synthesized fragment	Chemically synthesized fragment
Forward primers			
F1	CATGCTGTCTAACATGGAATCTGT	CATGATGGCGTGGAAAGATTG	CATGGATCTGTCTAACATGGAC
F2	CTGTCTAACATGGAATCTGTAGTAG	ATGGCGTGGAAAGATTGCATTATT	GAGAATCTTTATTTTCAGGGCGCGTGG AAAGATTGCATTA GATCTGTCTAACATGGAATCTGT
Reverse primer			
R1	GCTACATAGTGCTGCTCGGGTT	GACAGGATTCGTCATCGTCT	CCCTGAAATAAAGATTCTCCATAGTG CTGCACGGGTTA
R2	TCGAGCTACATAGTGCTGCACGGGT	TCGAGACAGGATTCGTC	TCGAGTCAACAGGATTCGTCATCG
R3			GATTCAACAGGATTCGTCATCGTCTG
Cloning vector	Modified pETM-13	Modified pET-28a	Modified pET-GB1-1a
Expression host	<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> BL21 (DE3)	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MMLSNMESVVESALTGQRTKIIVKVHM PCGKSRKAMALAASVNGVDSVEITG EDKDLVVVGRGIDPVRLLVALLREKC GLAELLMVELVEKEKTQLAGGKKGAY KKHPTYNLSPFDYVEYPPSAPIMQDI NPCSTM	MAWKDCIIQRYKDGVDNNIYTANRNEE ITIEEYKVFVNEACHPYVPILPDRSV LSGDFTSAYADDESC	MKHHHHHHPHMLSNMESVVESALTGQR TKIVVKVHMPGKSRKAMALAASSV NGVDSVEITGEDKDLVVGRGIDPVR LALLREKCGLAELLMVELVEKEKTQL AGGKKGAYKKHPTYNLSPFDYVEYPP SAPIMQDINPCSTMSSGSGSENLYFO GAMD AWKDCIIQRYKDGVDNNIYTAN RNEEITIEEYKVFVNEACHPYVPILP DRSVLSGDFTSAYADDESC LEHHHH HH†

† Blue and red colours denote the amino-acid sequences of RGA5A_S and AVR1-CO39, respectively. The TEV linker and 6×His tags are underlined.

domain and leucine-rich repeat proteins (NLRs; Liu *et al.*, 2014). Only 11 corresponding *Avr* genes from *M. oryzae* have been cloned (Pieterse *et al.*, 2014; Devanna *et al.*, 2014; Wu *et al.*, 2015; Yoshida *et al.*, 2009). In several cases, rice-blast resistance has been shown to be conferred not by individual NLR proteins but by functional pairs of NLR proteins. The two NLR proteins in each pair cooperate to exert the pathogen-recognition and signalling functions (Zhai *et al.*, 2014; Kanzaki *et al.*, 2012; Cesari *et al.*, 2013).

RGA4 and RGA5 form a well studied NLR pair encoded by the *Pia* resistance gene in rice (Cesari *et al.*, 2013). RGA4 acts as a constitutive cell-death inducer, while RGA5 acts as an effector-binding receptor and a repressor of RGA4 signalling activity. RGA5 forms a heterocomplex with RGA4 to prevent cell death mediated by RGA4 in the absence of the effector AVR1-CO39. The effector physically interacts with an effector-binding domain of RGA5 (RGA5A_S, including a heavy-metal-associated domain; HMA) to relieve this suppression, resulting in a resistance response and HR (Cesari *et al.*, 2013, 2014). However, the precise function of the HMA domain in direct molecular recognition of the effector is still unclear in plant NLR biology. Little is known about how plant-pathogen effectors are directly recognized by NLRs at the molecular level. Another NLR pair in rice (Pik-1/Pik-2) recognizes the AVR1-PikD effector of *M. oryzae* via an HMA domain (Pikp-HMA). The crystal structure of the Pikp-HMA–AVR1-PikD complex has been resolved, and the structure revealed the details of the protein–protein molecular interactions involved in effector recognition (Maqbool *et al.*, 2015). It remains unknown whether the interaction between RGA5A_S and AVR1-CO39 takes place according to the same model as that involving Pikp-HMA and AVR1-PikD.

In this study, the binding affinity of AVR1-CO39 for RGA5A_S was determined using isothermal titration calorimetry (ITC) in order to further verify the formation of RGA5A_S–AVR1-CO39 complexes prior to crystallization. The complexes were prepared using two different methods and the purified samples were crystallized under two different conditions. We believe that further structural characterization of the interaction between RGA5A_S and AVR1-CO39 will lead to a better understanding of the mechanism underlying effector recognition by NLR proteins.

2. Materials and methods

2.1. Generation of expression constructs

The nucleotide sequences encoding RGA5A_S (residues Leu982–Met1116) and AVR1-CO39 (residues Ala22–Cys89, lacking the signal peptide) were codon-optimized for expression in *Escherichia coli* and then chemically synthesized. Polymerase chain reaction products were generated using sticky-end cloning (Pham *et al.*, 1998) using the primers shown in Table 1 and were then inserted into the pETM-13 and pET-28a expression vectors (kindly supplied by Dr Arie Geerloff, EMBO, Heidelberg, Germany) using the *Nco*I and *Xho*I restriction sites. The constructs supported the expression of an RGA5A_S protein without a fusion tag and an AVR1-CO39 protein with a C-terminal 6×His tag. The homologous recombination method (Raymond *et al.*, 1999) was used to subclone both RGA5A_S and AVR1-CO39 into the pET-GB1 expression vector (kindly supplied by Dr Arie Geerloff, EMBO, Heidelberg, Germany) to generate a tandem fusion protein with an N-terminal 6×His tag and a cleavage site

Table 2
Crystallization information.

	6×His-RGA5A_S-TEV-AVR1-CO39	Mixture of RGA5A_S and AVR1-CO39-6×His
Method	Sitting-drop vapour diffusion	Sitting-drop vapour diffusion
Plate type	96-well MRC plates	96-well MRC plates
Temperature (K)	289	289
Protein concentration (mg ml ⁻¹)	3.36	5.6 (RGA5A_S), 12 (AVR1-CO39)
Buffer composition of protein solution	20 mM Tris–HCl pH 8, 150 mM NaCl, 5 mM DTT, 4 mM EDTA	20 mM Tris–HCl pH 8, 150 mM NaCl, 5 mM DTT, 4 mM EDTA
Composition of reservoir solution	2 M NaCl	0.1 M sodium acetate–HCl pH 4.6, 1.1 M ammonium tartrate dibasic
Volume and ratio of drop	0.5 µl, 7:3 protein solution:reservoir solution	0.5 µl, 7:3 protein solution:reservoir solution
Volume of reservoir (µl)	35	35

Figure 1
Formation of the fusion complex of RGA5A_S and AVR1-CO39. (a) Analytical gel-filtration traces depicting the retention fractions of RGA5A_S-TEV-AVR1-CO39. Black dotted line, protein molecular-weight marker (Superdex 75 10/300 GL); red line, RGA5A_S-TEV-AVR1-CO39. Protein elution was monitored by measuring the optical absorbance at 280 nm. The inset shows sodium dodecyl sulfate polyacrylamide gel electrophoresis results for the elution peak fractions. (b) The formation of the fusion complex RGA5A_S-TEV-AVR1-CO39 was characterized by SV-AUC. Sedimentation-velocity $c(s)$ functions were derived from sedimentation-velocity data.

(6×His-RGA5A_S-TEV-AVR1-CO39). The TEV cleavage site was incorporated to produce a fusion protein with a longer flexible linker to facilitate cleavage to obtain the two components. All constructs were verified by DNA sequencing.

2.2. Protein expression and purification

All recombinant proteins were overexpressed in *E. coli* BL21 (DE3) cells. The cells were grown in lysogeny broth in a shaking incubator at 310 K. When the optical density at 600 nm (OD_{600}) reached 0.6–0.8, protein expression was induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside and the cells were further incubated at 291 K for 18–20 h. The cells were collected by centrifugation at 6000 rev min⁻¹ for 10 min at 277 K and were then resuspended in 60–80 ml lysis buffer (20 mM Tris–HCl pH 8, 500 mM NaCl). The cell pellets were disrupted by ultrasonication and the debris was removed by centrifugation at 12 000 rev min⁻¹ for 30 min at 277 K. The lysates were first purified using a nickel-chelating Sepharose Fast Flow agarose column. The bound proteins were step-eluted with elution buffer (lysis buffer containing 500 mM imidazole). The eluted fractions were concentrated and further purified by chromatography using Superdex 75 10/300 GL gel-filtration columns (GE Healthcare, Little Chalfont, England) that had been equilibrated in gel-filtration buffer [20 mM Tris–HCl pH 8, 150 mM NaCl, 5 mM dithiothreitol (DTT), 4 mM ethylenediaminetetraacetic acid (EDTA)]. The peak fractions were pooled and concentrated to 4–12 mg ml⁻¹. All experimental steps were performed at 277 K.

2.3. Analytical ultracentrifugation

Sedimentation-velocity analytical ultracentrifugation (SV-AUC) was carried out in a Beckman Coulter XL-I analytical ultracentrifuge to determine complex formation by the fusion protein RGA5A_S-TEV-AVR1-CO39. The fusion complex was dissolved at 1 mg ml⁻¹ in 20 mM Tris–HCl pH 8.0, 150 mM NaCl. The complex was centrifuged using an An-50Ti rotor at a speed of 40 000 rev min⁻¹ and a rotor temperature of 20°C. Data were collected using interference optics and the related sedimentation-coefficient distribution $c(s)$ was calculated using the *SEDFIT* software (Brown & Schuck, 2006).

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Proteins for crystallization	Mixture of RGA5A_S and AVR1-CO39-6×His	6×His-RGA5A_S- TEV-AVR1-CO39
Diffraction source	BL18U1, SSRF	BL17U, SSRF
Wavelength (Å)	0.9795	0.9793
Temperature (K)	100	100
Detector	Dectris PILATUS3 6M	ADSC Q315r
Crystal-to-detector distance (mm)	350	250
Rotation range per image (°)	1	1
Total rotation range (°)	240	240
Exposure time per image (s)	1	1
Space group	<i>P</i> 3 ₁ 21	<i>I</i> 4
<i>a</i> , <i>b</i> , <i>c</i> (Å)	66.2, 66.2, 108.8	137.4, 137.4, 66.2
α , β , γ (°)	90, 90, 120	90, 90, 90
Mosaicity (°)	0.20	0.42
Resolution range (Å)	39.47–2.108 (2.184–2.108)	31.32–2.703 (2.800–2.703)
Total No. of reflections	215689 (21441)	164731 (24327)
No. of unique reflections	16480 (1605)	17109 (247)
Completeness (%)	100 (99)	100 (100)
Multiplicity	13.1 (13.4)	11.5 (2.5)
$\langle I/\sigma(I) \rangle$	10.11 (3.88)	11.5 (2.50)
$R_{\text{r.i.m.}}$ [†]	0.1878 (0.593)	0.142 (1.203)
Overall <i>B</i> factor from Wilson plot (Å ²)	44.15	67.51

$$^{\dagger} R_{\text{r.i.m.}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl).$$

2.4. ITC

ITC experiments were performed at 298 K using a MicroCal iTC₂₀₀ instrument (Malvern Instruments, Malvern, England). The purified proteins were dissolved in 20 mM Tris–HCl pH 8, 150 mM NaCl. 20 injections of AVR1-CO39 (420 μ M) in the injection syringe were titrated by adding 2 μ l at a time to a 300 μ l sample cell containing 40 μ M RGA5A_S. The stirring rate was 750 rev min^{−1} and the dynamics of the thermal power were recorded every 5 s. The heat-exchange data for all of the injections were fitted to a single-site binding model using the *Origin* software suite (OriginLab, Northampton, Massachusetts, USA).

2.5. Crystallization

RGA5A_S and AVR1-CO39-6×His were mixed in a 1:1 molar ratio and the mixture was incubated on ice overnight prior to cocrystallization. The initial crystallization screening was based on the crystallization conditions for RGA5A_S (Huang *et al.*, 2015). The proteins were dissolved in 20 mM NaH₂PO₄/Na₂HPO₄ pH 7, 500 mM NaCl, 1 mM DTT. Initial crystallization was based on the sitting-drop vapour-diffusion method using an Oryx4 crystallization robot (Douglas Instruments, Hungerford, England) with 0.2 M ammonium citrate, 25% (w/v) PEG 3350. Drops (0.5 μ l) were prepared by diluting the protein complex solution to various concentrations using the reservoir solution and then equilibrating the drops against 35 μ l well solution. The compositions of the solutions are shown in Table 2. However, no crystals grew. The crystallization conditions for the two complexes were further

screened using the MCSG-1, MCSG-2, MCSG-3 and MCSG-4 screens from Microlytic (<http://www.microlytic.com>) using a random-screening strategy. The final crystallization conditions for both complexes are shown in Table 2.

2.6. Data collection and structure determination

The crystals were first transferred into reservoir solution supplemented with an additional 20% (w/v) glycerol as a cryoprotectant and were then flash-cooled by plunging them into liquid nitrogen. Diffraction data were collected at 100 K on beamline BL18U at the Shanghai Synchrotron Research Facility (SSRF). The data were indexed and scaled with *xia2* as implemented in the *CCP4* suite (Winn *et al.*, 2011; Winter, 2010). The initial model was built by the molecular-replacement method with *Phaser* (McCoy *et al.*, 2007) using the structures of RGA5A_S (unpublished work) and Pikp-HMA/AVR1-PikD (PDB entry 5a6w; Maqbool *et al.*, 2015) as search

Figure 2
Isothermal titration calorimetry (ITC) measurements of the interaction between AVR1-CO39 and RGA5A_S. The upper panel shows raw data for AVR1-CO39 titrated with RGA5A_S. The area of each injection peak corresponds to the total heat release for that injection. The bottom panel shows the integrated heat release for all injections (squares) and the best fit (solid line) to a single-site binding model using the *Origin* software suite.

models. The data-collection and processing statistics for both structures are listed in Table 3.

3. Results and discussion

Three strategies are commonly used to prepare protein complexes for crystallization. In the first strategy, each individual protein in the complex is purified separately and the purified proteins are mixed to form the complex prior to crystallization (mixing strategy). In the second approach, plasmids that express protein A with a fusion tag and protein B without a tag are cotransformed into a microbial strain for protein expression; these are then purified together to form the complex (known as the coexpression strategy; Ke *et al.*, 2015; Maqbool *et al.*, 2015). A third, linker-based strategy fuses the two proteins together to prepare tandem complex proteins (Lucato *et al.*, 2015; Morita *et al.*, 2017). The second and third strategies are often used to prepare specific proteins

with properties such as poor stability, low expression levels or weak binding affinity.

We failed to obtain soluble proteins using the co-expression strategy (data not shown); however, RGA5A_S, AVR1-CO39 and the fusion complex RGA5A_S-TEV-AVR1-CO39 were efficiently expressed in *E. coli* BL21 (DE3) cells. Using similar purification processes, the three proteins were first purified using nickel-chelating Sepharose Fast Flow agarose, followed by gel filtration using a Superdex 75 10/300 GL column. AVR1-CO39 was in a homogeneous state. In contrast, RGA5A_S showed a wide elution range from the gel-filtration column. For the fusion complex, the elution volume of 10.6 ml from this column corresponded to a molecular weight of 37.7 kDa, which was close to the expected molecular weight for the tandem complex (26.5 kDa; Fig. 1*a*). SV-AUC was carried out in a Beckman Coulter XL-I analytical ultracentrifuge to determine the molecular weight of the RGA5A_S-TEV-AVR1-CO39 fusion complex. The SV-AUC data showed that the major component had a sedimentation

Figure 3
RGA5A_S-AVR1-CO39 complex crystals. (a) Crystal of the RGA5A_S-AVR1-CO39 complex obtained using reservoir solution consisting of 0.1 M sodium acetate-HCl pH 4.6, 1.1 M ammonium tartrate dibasic. (b) The tandem complex of RGA5A_S-TEV-AVR1-CO39 obtained using 2 M NaCl. The upper panels show the crystals and the lower panels show the corresponding diffraction images.

coefficient of 2.413 S (~ 23.7 kDa), corresponding to a monomer of the complex (26.5 kDa; Fig. 1b). These results indicate that the fusion complex formed a monomer in solution.

The binding affinity between RGA5A_S and AVR1-CO39 was determined by isothermal titration calorimetry (Fig. 2) to further verify the formation of an RGA5A_S–AVR1-CO39 complex prior to cocrystallization. In agreement with previously reported yeast two-hybrid results (Césari *et al.*, 2013, 2014), the ITC data showed that there is specific, direct binding with a one-site model and an equilibrium dissociation constant (K_d) of 5.4 μ M for RGA5A_S and AVR1-CO39.

The RGA5A_S–AVR1-CO39 complex (complex I) was prepared by mixing the proteins together in a 1:1 molar ratio and was successfully crystallized after two or three weeks in 0.1 M sodium acetate–HCl pH 4.6, 1.1 M ammonium tartrate dibasic (Fig. 3a). Additionally, the TEV cleavage site in the fusion complex was not cleaved after purification and crystals of the fusion complex were grown in reservoir solution consisting of 2 M NaCl after 70 d (Fig. 3b). The two crystals had different forms and diffracted to Bragg spacings of 2.4 and 2.72 Å resolution.

The initial models were constructed by the molecular-replacement method using *Phaser* (McCoy *et al.*, 2007). For complex I, the crystal belonged to space group $P3_121$, with unit-cell parameters $a = 66.2$, $b = 66.2$, $c = 108.8$ Å. The calculated Matthews coefficient indicated that there was one molecule of RGA5A_S and one molecule of AVR1-CO39 in the asymmetric unit, with an approximate solvent content of 64.2%. Using *Phaser* (McCoy *et al.*, 2007), a solution with a rotation-function Z-score of 6.3, a translation-function Z-score of 33.2 and a convincing log-likelihood gain of 1021 was identified. Subsequently, the initial structure was refined using *PHENIX* (Adams *et al.*, 2010), which resulted in a model with an R_{work} of 34.6% and an R_{free} of 36.9%. The crystal of the fusion complex belonged to space group $I4$, with unit-cell parameters $a = 137.4$, $b = 137.4$, $c = 66.2$ Å. There were two molecules of RGA5A_S and two molecules of AVR1-CO39 in the asymmetric unit, with a solvent content of 68.5%. The molecular-replacement solution had rotation-function and translation-function Z-scores of 3.4 and 15, respectively, and a log-likelihood gain of 1729. Initial refinement of the structure resulted in a model with an R_{work} of 28.5% and an R_{free} of 30.6%.

Structure refinement is currently ongoing. A detailed structural analysis of the interaction between RGA5A_S and AVR1-CO39 should help us to determine the molecular mechanism by which the pathogen is directly recognized by the host. The result also shows that different strategies should be employed to prepare complexes to increase the success rate of crystallization.

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