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Purification, crystallization and preliminary X-ray crystallographic analysis of a rice Rac/Rop GTPase, OsRac1

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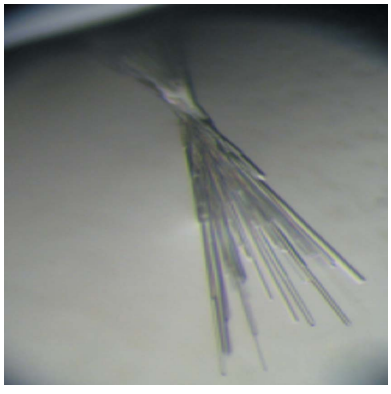

Small GTPases regulate a large variety of key cellular processes. Plant small Rac/Rop GTPases have recently received broad attention as it is becoming clear that these enzymes regulate various plant cellular processes. OsRac1, a rice Rac/Rop protein, is a key regulator of reactive oxygen species (ROS) production and induces immune responses. Although four structures of plant small GTPases have been reported, all of these were of the inactive form. Here, OsRac1 was purified and co-crystallized with the GTP analogue 5′-guanylyl imidodiphosphate (GMPPNP). The crystal belonged to space group $P2_12_12_1$ and a complete data set was collected to 1.9 Å resolution.

1. Introduction

The Ras superfamily of small GTPases (20–30 kDa) comprises five functional families of GTPases referred to as Ras, Ran, Rab, Arf and Rho/Rac. The interconversion between GDP-bound (inactive) and GTP-bound (active) forms allows Ras superfamily members to regulate a number of cellular processes by means of a molecular-switch mechanism for extracellular signals in eukaryotes. Although plants lack Ras family and typical animal Rho family members, they contain the Rho-related Rac/Rop GTPases (Nibau *et al.*, 2006; Nagawa *et al.*, 2010). Like other small GTPases, Rac/Rop family members function as molecular switches by interconversion between inactive GDP-bound and active GTP-bound forms in cells, and are an important regulator of signal transduction (Nibau *et al.*, 2006; Mucha *et al.*, 2011; Craddock *et al.*, 2012).

In rice, OsRac1 (Rop of *Oryza sativa*) initiates defence responses through activation of the NADPH-mediated production of reactive oxygen species (ROS) by direct binding to the plant NADPH oxidase OsRbohB (Rboh; respiratory burst oxidase homologue; Kawasaki *et al.*, 1999; Wong *et al.*, 2007; Oda *et al.*, 2010). OsRac1-induced immune responses result in cell death as well as disease resistance against rice-blast fungus, rice bacterial blight and *Tobacco mosaic virus* (Kawasaki *et al.*, 1999; Ono *et al.*, 2001; Moeder *et al.*, 2005). Moreover, the constitutively active form of OsRac1 interacts strongly with the nucleotide-binding (NB) domain of the rice intracellular immune receptor Pit, and OsRac1 regulates downstream of Pit in the signalling pathways (Kawano *et al.*, 2010). Although it is known that OsRac1 plays important roles as a molecular switch in the innate immunity of rice, details of the molecular-switch mechanism involved remain largely unknown.

Here, the purification, crystallization and preliminary crystallographic study of activated OsRac1 are reported. A specific mutation (Gln68 to Leu) of the protein was employed and this mutant was co-crystallized with the GTP analogue 5′-guanylyl imidodiphosphate (GMPPNP) in order to maintain OsRac1 in the active form.

2. Materials and methods

2.1. Macromolecule production

2.1.1. Protein expression. The *O. sativa* Rac1 gene (OsRac1; residues 8–183) was cloned into the pGEX6P-3 vector (GE Health-

Table 1
Data-collection statistics.

Values in parentheses are for the outer shell.

Wavelength (Å)	0.9000
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 36.8, b = 59.1, c = 64.4
Resolution range (Å)	50.0–1.9 (1.97–1.90)
Total No. of reflections	93928
No. of unique reflections	11521
Completeness (%)	99.8 (100.0)
Multiplicity	8.2 (8.3)
$I/\sigma(I)$	19.7 (7.7)
$R_{\rm merge}$†	0.095 (0.284)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I_i(hkl)\rangle$ is the average intensity obtained from multiple observations of symmetry-related reflections.

care) using *Bam*HI and *Xho*I restriction-enzyme sites. Point mutations (C32S, G19V and Q68L) were performed using the QuikChange site-directed mutagenesis kit (Stratagene). The nonconserved surface cysteine residue (Cys32) was substituted by serine to increase the stability of the protein. The OsRac1(8–183) C32S/Q68L (referred to as OsRac1-Q68L) or C32S/G19V (referred to as OsRac1-G19V) proteins were expressed in *Escherichia coli* Rosetta (DE3) cells (Novagen) using M9 minimal medium and were induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) at an optical density (OD_{600}) of 0.5. Following incubation at 288 K for 12 h, the cells were harvested by centrifugation at 4000*g* for 20 min at 277 K.

2.1.2. Purification. The cells were suspended in lysis buffer (50 m*M* Tris–HCl pH 7.5, 400 m*M* NaCl, 5 m*M* $MgCl_2$, 2 m*M* DTT) and lysed by sonication on ice. The supernatant was collected by ultracentrifugation (110 000*g* for 30 min at 277 K) and then applied onto a Glutathione Sepharose 4B column (GE Healthcare). The column was washed with lysis buffer and the glutathione *S*-transferase tag was removed by on-column digestion at 277 K overnight with HRV 3C protease. The cleaved protein was eluted and purified by gel-filtration chromatography using a HiLoad 26/600 Superdex 75 column (GE Healthcare) pre-equilibrated with buffer *A* (50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 10 m*M* EDTA, 2 m*M* DTT). Under denaturing and reducing conditions, the SDS–PAGE showed a single band at about 20 kDa for OsRac1-Q68L.

2.2. Crystallization

For nucleotide exchange, purified OsRac1 (1–2 mg ml^{-1}) was incubated with a 25-fold excess of GMPPNP (Sigma) in buffer *A* for 12 h at 277 K. Following the addition of 10 m*M* $MgCl_2$, excess GMPPNP was removed by passage through a HiLoad 26/600 Superdex 75 column (GE Healthcare) in buffer *B* (10 m*M* Tris–HCl pH 7.5, 50 m*M* NaCl, 5 m*M* $MgCl_2$, 2 m*M* DTT). The protein was then concentrated to 4 mg ml^{-1} by ultrafiltration (10 kDa molecular-weight cutoff; Amicon Ultra, Millipore) in buffer *B*. The homogeneity of the purified protein was confirmed by AutoFlex MALDI–TOF MS (Bruker Daltonics) or SDS–PAGE using a buffer consisting of 40 m*M* Tris–HCl pH 6.8, 0.4% SDS, 0.4% β-mercaptoethanol, 0.2% bromophenol blue, 20% glycerol, 9 *M* urea, 50 m*M* DTT, 10 m*M* EDTA.

Initial crystallization screening was carried out at 277 K by the sitting-drop vapour-diffusion method in a 96-well crystallization plate (Hampton Research) using commercial screening kits including Crystal Screen, Crystal Screen 2, PEG/Ion, Index, Grid Screen PEG 6000, Grid Screen Ammonium Sulfate (Hampton Research) and The JCSG Core Suites (Qiagen). Crystals of OsRac1-Q68L in complex with GMPPNP were grown at 293 K by the sitting-drop vapour-diffusion method by mixing 0.7 µl of a 4 mg ml^{-1} protein solution in buffer *B* with 0.7 µl reservoir solution consisting of 100 m*M* MES buffer pH 6.0, 10–30% PEG 6000.

2.3. Data collection and processing

For the X-ray diffraction experiments, crystals were flash-cooled in a 100 K dry nitrogen stream using reservoir solution supplemented with 25%(*v*/*v*) glycerol as a cryoprotectant. Diffraction data were collected from native crystals of OsRac1 using a Rayonix MX225HE CCD detector installed on the BL44 beamline at SPring-8, Harima, Japan. The oscillation width was 1.5° per image and 160 images were collected. All data were processed and scaled using the *HKL*-2000 program suite (Otwinowski & Minor, 1997). Data-collection and scaling statistics are given in Table 1.

3. Results and discussion

3.1. Preparation

In order to characterize the structure of OsRac1 in the GTP-bound active state, we introduced a specific mutation (Gln68 to Leu) as reported for the human small GTPase RhoA (Longenecker *et al.*, 2003). In the RhoA protein, the single mutation Q63L (Q68L in OsRac1) results in a marked decrease in the GTP hydrolysis activity, thereby maintaining the protein in a constitutively active form (Longenecker *et al.*, 2003). The OsRac1-Q68L protein was prepared from bacterial cultures and purified chromatographically (Fig. 1*a*). The nucleotide bound to the purified protein was exchanged for the nonhydrolyzable GTP analogue GMPPNP. In the gel-filtration analysis, OsRac1-Q68L containing GMPPNP eluted at a similar volume as myoglobin (~17 kDa; Fig. 1*b*, marked *D*), suggesting that OsRac1-Q68L is a monomer in solution.

The OsRac1-G19V and wild-type OsRac1 proteins were also prepared for comparison with OsRac1-Q68L. The G19V (Gly19 to Val) mutation was introduced into OsRac1 since it has been shown

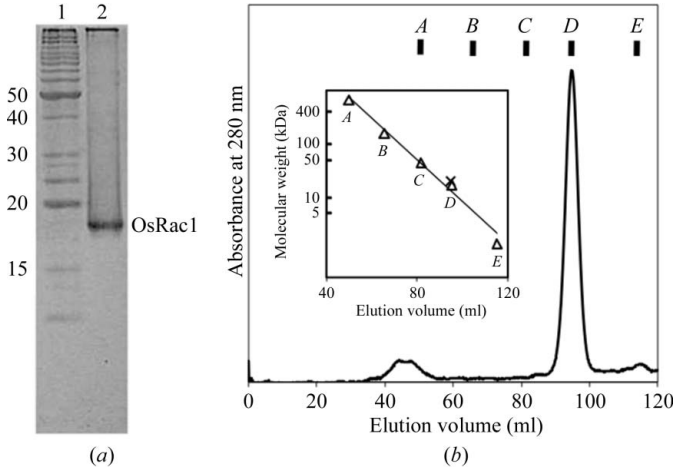

Figure 1
(*a*) 15% SDS–PAGE stained with Coomassie Brilliant Blue. Lanes 1 and 2 contain molecular-weight marker (labelled in kDa) and purified OsRac1-Q68L, respectively. (*b*) Elution profile of gel filtration of OsRac1-Q68L. The inset shows the calibration curve. The open rectangles correspond to the following marker proteins: *A*, thyroglobulin, 670.0 kDa; *B*, γ-globulin, 158.0 kDa; *C*, ovalbumin, 44.0 kDa; *D*, myoglobin, 17.0 kDa; *E*, vitamin B_{12}, 1.4 kDa. The cross represents the peak-top position of OsRac1, assuming the molecular weight of the monomeric form containing GMPPNP and Mg^{2+} ion. MALDI–TOF MS analysis gave a single peak at 19 905.8 Da corresponding to the calculated molecular mass of OsRac1-Q68L (19 963.9 Da).

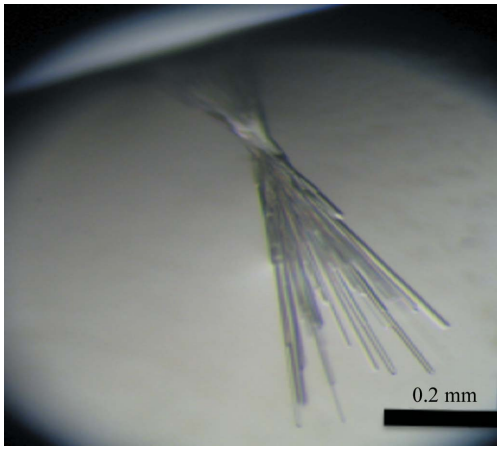

Figure 2
Crystals of OsRac1-Q68L in complex with GMPPNP. The scale bar represents 0.2 mm.

that a G14V (G19V in OsRac1) mutation in RhoA results in a marked decrease in the GTP hydrolysis activity (Longenecker *et al.*, 2003), thereby maintaining the protein in a constitutively active form in rice (Kawasaki *et al.*, 1999). Although both the OsRac1-G19V and wild-type OsRac1 proteins were successfully purified and converted to GMPPNP-bound forms, crystals of these proteins have yet to be obtained.

3.2. Data collection and phase determination

The asymmetric unit of the crystal (Fig. 2) contained one OsRac1 molecule, corresponding to a Matthews coefficient V_M of 1.75 Å^3 Da^{-1} (Matthews, 1968). Since OsRac1 shows 76% sequence identity to *Arabidopsis thaliana* Rac7 (AtRac7), the structure of AtRac7–GDP (PDB entry 2j0v; Sørmo *et al.*, 2006) was considered to be a suitable search model for use in the molecular-replacement approach. Our study should be helpful in obtaining the structure of the active form of OsRac1, thereby contributing towards efforts to delineate the mechanism of immunity responses facilitated by OsRac1 activation.

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