

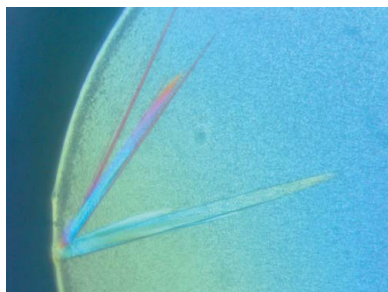
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Received 31 January 2012
Accepted 2 March 2012



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Purification, crystallization and preliminary X-ray analysis of OsAREB8 from rice, a member of the AREB/ABF family of bZIP transcription factors, in complex with its cognate DNA

The AREB/ABF family of bZIP transcription factors play a key role in drought stress response and tolerance during the vegetative stage in plants. To reveal the DNA-recognition mechanism of the AREB/ABF family of proteins, the bZIP domain of OsAREB8, an AREB/ABF-family protein from *Oryza sativa*, was expressed in *Escherichia coli*, purified and crystallized with its cognate DNA. Crystals of the OsAREB8–DNA complex were obtained by the sitting-drop vapour-diffusion method at 277 K with a reservoir solution consisting of 50 mM MES pH 6.4, 29% MPD, 2 mM spermidine, 20 mM magnesium acetate and 100 mM sodium chloride. A crystal diffracted X-rays to 3.65 Å resolution and belonged to space group *C*222, with unit-cell parameters $a = 155.1$, $b = 206.7$, $c = 38.5$ Å. The crystal contained one OsAREB8–DNA complex in the asymmetric unit.

1. Introduction

The plant hormone abscisic acid (ABA) controls drought stress response and tolerance in plants. ABA induces many drought-responsive genes in plants (Yamaguchi-Shinozaki & Shinozaki, 2006; Nakashima *et al.*, 2009; Fujita *et al.*, 2011). In the ABA signalling pathway ABA is recognized by its receptors, PYL/PYR/RCAR proteins (Ma *et al.*, 2009; Park *et al.*, 2009), and the ABA–receptor complex inhibits the phosphatase activity of the group A protein phosphatases 2C (PP2Cs) by direct interaction (Miyazono *et al.*, 2009; Melcher *et al.*, 2009). The inhibition of PP2Cs results in the activation of subclass III protein kinases of the SNF1-related protein kinases 2 (SnRK2s). The activated SnRK2s phosphorylate downstream substrates such as the AREB/ABF family of bZIP transcription factors to activate ABA-responsive gene expression.

AREB1 is one of the AREB/ABF-family transcription factors in *Arabidopsis thaliana*, which are positive regulators of ABA signalling involved in drought stress tolerance (Uno *et al.*, 2000; Fujita *et al.*, 2005; Furihata *et al.*, 2006; Yoshida *et al.*, 2010). AREB1 possesses four conserved phosphorylation sites (C1–C4), which are the targets of SnRK2s, and a bZIP-type DNA-binding domain between the C3 and C4 phosphorylation sites. AREB1 from *Arabidopsis* belongs to a group A subfamily of bZIP (basic region leucine zipper) groups (Jakoby *et al.*, 2002). In this group, the amino-acid sequences of the four conserved phosphorylation sites and the bZIP-type DNA-binding domain are highly conserved. AREB1 is activated by ABA-dependent multisite phosphorylation of the conserved domain (Furihata *et al.*, 2006) in order to recognize a conserved *cis*-element (designated the ABA-responsive element; ABRE; PyACGTGG/TC) which exists in the promoters of ABA-inducible genes (Giraudat *et al.*, 1994; Busk & Pagès, 1998).

The bZIP domain of the AREB/ABF family possesses three or four leucine repeats in its C-terminal leucine-zipper region and a highly conserved N-terminal basic region for the recognition of the ABRE sequence. In general, bZIP domains recognize divergent DNA sequences using five conserved signature sequences in the basic

region (Fujii *et al.*, 2000; Fig. 1). For example, the CREB and Fos/Jun families have an N₁XXA₄A₅XXC₈R₉ sequence (where X represents a variable amino-acid residue) in these basic regions for the recognition of CREB and AP-1 sites, respectively. In contrast, the AREB/ABF family has an N₁XXS₄A₅XXS₈R₉ sequence in its basic region for the recognition of the ABRE sequence. Although the Ser residue at position 8 is also observed in other bZIP families, such as the PAR and C/EBP families, the Ser residue at position 4 is unique to the AREB/ABF family. Although the structures of CREB, Fos/Jun and C/EBP in complex with these cognate DNAs have been reported (Glover & Harrison, 1995; Schumacher *et al.*, 2000; Miller *et al.*, 2003), the structure of the AREB/ABF family is still unknown. The rice AREB/ABF-family transcription factor OsAREB8 (Os06g0211200), which is also known as OsbZIP46, OsABF2 and ABL1, regulates ABRE-dependent gene expression in response to ABA, auxin and several abiotic stress responses such as drought and oxidative stresses (Nijhawan *et al.*, 2008; Hossain *et al.*, 2010; Yoshida *et al.*, 2010; Yang *et al.*, 2011). To elucidate the structural basis of the DNA-binding mechanism of the AREB/ABF family of bZIP proteins, the bZIP domain of OsAREB8 was expressed in *Escherichia coli*, purified and crystallized with a double-stranded DNA containing the ABRE sequence.

2. Materials and methods

2.1. Overexpression, purification and crystallization

The gene fragment for the OsAREB8 bZIP domain (residues 235–308 of OsAREB8) was amplified by PCR and cloned into the *Nde*I/*Hind*III site of pET-26b plasmid (Novagen). The constructed plasmid (pET26b-OsAREB8) was transformed into *E. coli* strain Rosetta (DE3) to express OsAREB8. The transformants were cultured in LB medium supplemented with 20 µg ml⁻¹ kanamycin at 310 K. OsAREB8 expression was induced by the addition of 1 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density of the medium at 600 nm reached 0.6. The cells were cultured for a further 14 h at 298 K. After cultivation, the cells were harvested by centrifugation at 5000g for 10 min. The harvested cells were resuspended in 10 mM Tris–HCl pH 7.5 and disrupted by sonication. To remove contaminant DNA derived from *E. coli*, 0.01% (v/v) (final concentration) polyethyleneimine was added to the disrupted solution. After centrifugation at 40 000g for 30 min, the supernatant was supplemented with 2 mM (final concentration) MgCl₂ and treated with Benzonase nuclease (Novagen). The treated solution was applied onto a TOYOPEARL AF-Heparin HC-650 (Tosoh) column. The column was washed with 10 mM Tris–HCl pH 7.5, 250 mM NaCl, and the bound OsAREB8 was eluted with 10 mM Tris–HCl pH 7.5, 1 M NaCl. The eluted protein was further purified

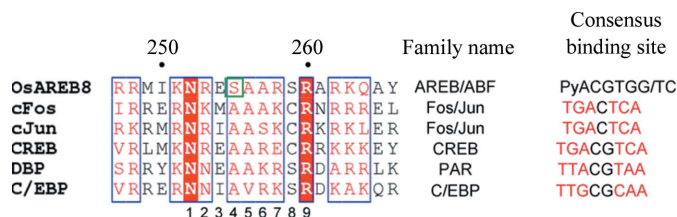


Figure 1

Amino-acid sequence comparison of the basic regions of bZIP transcription factors and their consensus sequences. The characteristic serine residue of OsAREB8 is indicated by a green square. The alignment figure was prepared using *ESPrpt* (Gouet *et al.*, 1999). The most conserved palindromic bases in the consensus binding sites are shown in red.

using a 6 ml Resource S (GE Healthcare) column pre-equilibrated with 10 mM Tris–HCl pH 7.5 and was eluted with a linear gradient of 0–1 M NaCl. The purified protein was dialyzed against 10 mM Tris–HCl pH 7.5, 50 mM NaCl for crystallization. All purification experiments were performed at 277 K.

For crystallization of the OsAREB8–DNA complex, 14 bp blunt-end double-stranded DNAs containing the ABRE sequence (5′-CTGCC**ACGTGGC**AG-3′, 5′-CTAT**ACGTGGC**AG-3′ and 5′-TGCC**ACGTGGC**AGG-3′; the ABRE sequence is indicated in bold) were added to the OsAREB8 solution. The OPC-purified oligonucleotides were purchased from Operon Biotechnology (Tokyo, Japan) and were annealed by heating to 368 K for 1 min and slow cooling to 277 K in 10 mM Tris–HCl pH 7.5, 50 mM NaCl. Because bZIP domains form a homodimer to recognize the DNA sequence, the purified OsAREB8 and the double-stranded DNA were mixed in a 2:1 molar ratio. The OsAREB8–DNA complex was concentrated to 500 µM for crystallization. All crystallization experiments were performed at 277 K using the sitting-drop vapour-diffusion method. Each drop was prepared by mixing 1 µl OsAREB8–DNA solution and 1 µl reservoir solution.

2.2. Data collection and processing

The crystals were mounted on cryoloops and flash-cooled in a nitrogen stream at 95 K for data collection. An X-ray diffraction data set was collected on the AR-NW12A beamline at the Photon Factory (PF; Tsukuba, Japan) using an ADSC Quantum 210r detector and an X-ray wavelength of 1.0000 Å. The diffraction data were indexed and integrated with the program *XDS* (Kabsch, 2010) and scaled with the program *SCALA* from the *CCP4* suite (Winn *et al.*, 2011). The anisotropy of the data was analyzed by the *Diffraction Anisotropy Server* (<http://services.mbi.ucla.edu/anisotropy/>; Strong *et al.*, 2006). A self-rotation function was calculated using the program *MOLREP* (Vagin & Teplyakov, 2010). The initial structure of the OsAREB8–DNA complex was determined by the molecular-replacement method using the program *MOLREP* with the coordinates of the MafG–DNA complex (PDB entry 3a5t; the amino-acid sequence identity of the MafG bZIP domain to OsAREB8 is 37%; Kurokawa *et al.*, 2009). A total of 5% of the reflections were randomly selected to provide a

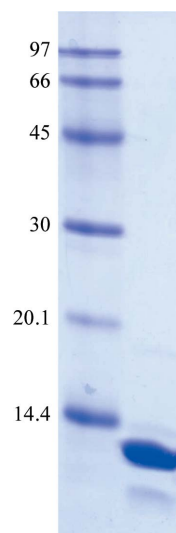


Figure 2

SDS-PAGE (15%) of OsAREB8. The approximate molecular weight of OsAREB8 (residues 235–308) is 8800.

test set for R_{free} calculations. The initial model was refined using the program *REFMAC5* (Murshudov *et al.*, 2011).

3. Results and discussion

Recombinant OsAREB8 was expressed in *E. coli* and purified by two column-chromatographic steps. We used DNase to remove contaminant DNA from *E. coli*. This treatment increased the yield of OsAREB8. DNase and digested DNA fragments should be removed by the two column-chromatographic steps. SDS-PAGE analysis revealed that the OsAREB8 was more than 95% pure (Fig. 2). We only obtained diffraction-quality crystals when we used double-stranded DNA with sequence 5'-CTGCCACGTGGCAG-3' for cocrystallization. The best crystal of the OsAREB8-DNA complex was obtained using a reservoir solution consisting of 50 mM MES pH 6.4, 29% MPD, 2 mM spermidine, 20 mM magnesium acetate, 100 mM NaCl (Fig. 3). Because the crystallization conditions

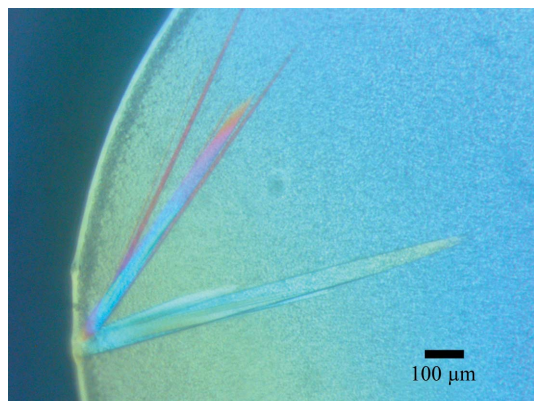


Figure 3
Crystals of OsAREB8 in complex with its cognate DNA.

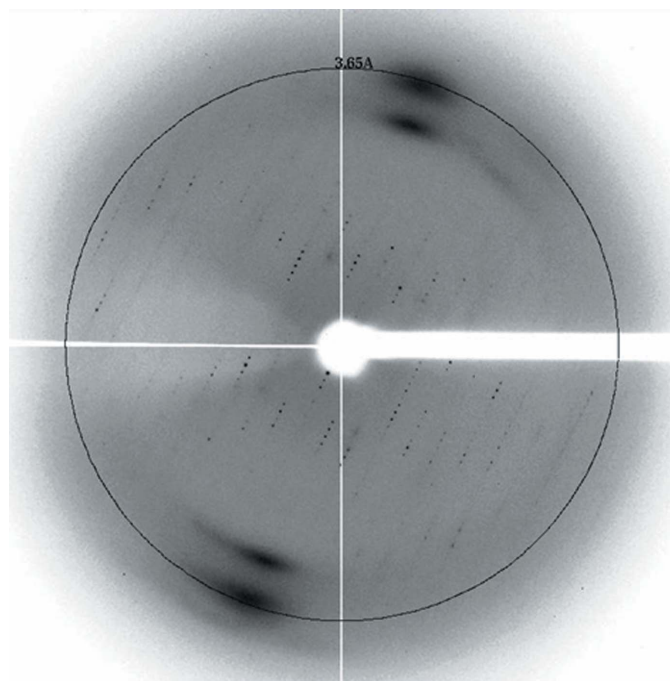


Figure 4
X-ray diffraction image of OsAREB8 in complex with its cognate DNA. The circle indicates a resolution of 3.65 Å.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Beamline	PF AR-NW12A
Wavelength (Å)	1.0000
Temperature (K)	95
Crystal-to-detector distance (mm)	301.4
Oscillation width (°)	1.0
Total No. of frames	180
Space group	C222
Unit-cell parameters	
<i>a</i> (Å)	155.1
<i>b</i> (Å)	206.7
<i>c</i> (Å)	38.5
Resolution range (Å)	20.0–3.65 (3.85–3.65)
Total No. of observations	51535 (7819)
No. of unique observations	7285 (1067)
Multiplicity	7.1 (7.3)
Completeness (%)	99.4 (100)
$R_{\text{merge}}^{\dagger}$ (%)	11.0 (78.4)
$\langle I/\sigma(I) \rangle$	13.4 (2.9)

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the i th intensity measurement of reflection hkl , including symmetry-related reflections, and $\langle I(hkl) \rangle$ is its average.

contained a high concentration of MPD, we did not use a cryoprotectant when we flash-cooled the crystals. The crystal of the OsAREB8-DNA complex diffracted X-rays to a resolution of 3.65 Å (Fig. 4) and belonged to space group *C222*, with unit-cell parameters $a = 155.1$, $b = 206.7$, $c = 38.5$ Å. Data-collection statistics are summarized in Table 1. The data set was severely anisotropic. Analysis using the *Diffraction Anisotropy Server* showed that the suggested diffraction limits ($F/\sigma < 3$) along the a^* and b^* directions were 4.6 and 4.1 Å, respectively, although the suggested diffraction limit along the c^* direction was 3.6 Å. In this study, the R_{merge} in the highest resolution shell was relatively high. This could be related to the severe anisotropy of the data.

According to Matthews coefficient analysis (Matthews, 1968), the crystal appeared to contain two or three OsAREB8-DNA complexes in the asymmetric unit. Assuming the presence of two or three complexes per asymmetric unit, the Matthews coefficient of the crystal would be 2.96 or 1.97 Å³ Da⁻¹, respectively. However, analysis of the self-rotation function did not reveal any significant peaks that indicate the presence of two or three complexes per asymmetric unit. In addition, the results of molecular replacement suggested that the

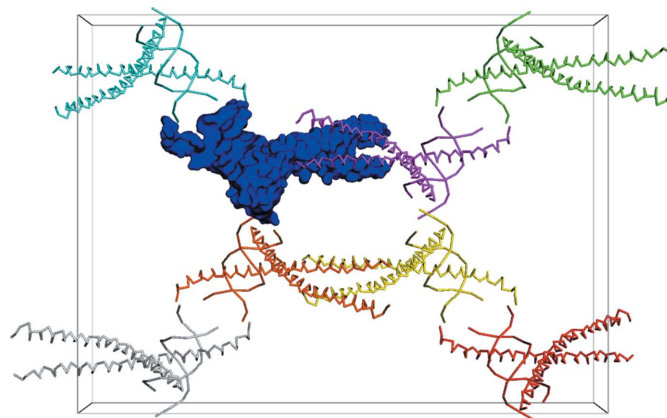


Figure 5
Crystal packing of the OsAREB8-DNA complex in the centred orthorhombic cell. The molecules in the asymmetric unit are shown as a darker blue surface, while symmetry-related molecules in the unit cell are shown as C^c traces. This image was created with *PyMOL* (DeLano, 2002).

crystal contained only one OsAREB8–DNA complex per asymmetric unit (Fig. 5). This means that the solvent content of the crystal of the OsAREB8–DNA complex is very high (the Matthews coefficient and solvent content of the crystal are $5.92 \text{ \AA}^3 \text{ Da}^{-1}$ and 79.25%, respectively). The *R* factor and *MOLREP* score after *MOLREP* were 0.626 and 0.545, respectively. The *R* factor, R_{free} and FOM values after 30 cycles of refinement by *REFMAC5* were 0.284, 0.377 and 0.596, respectively. Further model building and refinement are in progress.

The synchrotron-radiation experiments were performed on beamline AR-NW12A at the Photon Factory (Proposal No. 2008S2-001). This work was supported by the Targeted Proteins Research Program (TPRP) of the Ministry of Education, Culture, Sports, Science and Technology, Japan, by the Program for the Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN) and by the Science and Technology Research Partnership for Sustainable Development (SATREPS) of the Japan Science and Technology Agency (JST)/Japan International Cooperation Agency (JICA).

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