Crystallographic analysis of the *Arabidopsis thaliana* BAG5–calmodulin protein complex

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Arabidopsis thaliana BAG5 (AtBAG5) belongs to the plant BAG (Bcl-2-associated athanogene) family that performs diverse functions ranging from growth and development to abiotic stress and senescence. BAG family members can act as nucleotide-exchange factors for heat-shock protein 70 (Hsp70) through binding of their evolutionarily conserved BAG domains to the Hsp70 ATPase domain, and thus may be involved in the regulation of chaperone-mediated protein folding in plants. AtBAG5 is distinguished from other family members by the presence of a unique IQ motif adjacent to the BAG domain; this motif is specific for calmodulin (CaM) binding, indicating a potential role in the plant calcium signalling pathway. To provide a better understanding of the IQ motif-mediated interaction between AtBAG5 and CaM, the two proteins were expressed and purified separately and then co-crystallized together. Diffraction-quality crystals of the complex were grown using the sitting-drop vapour-diffusion technique from a condition consisting of 0.1 M Tris–HCl pH 8.5, 2.5 M ammonium sulfate. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 64.56$, $b = 74.89$, $c = 117.09$ Å. X-ray diffraction data were recorded to a resolution of 2.5 Å from a single crystal using synchrotron radiation. Assuming the presence of two molecules in the asymmetric unit, a Matthews coefficient of 2.44 Å³ Da^{−1} was calculated, corresponding to a solvent content of approximately 50%.

1. Introduction

Ca²⁺ acts as an important second messenger and regulates many cellular and developmental processes (Clapham, 2007). Particularly in plants, Ca²⁺ participates in mediating responses to hormones and environmental signals, including biotic (for example, pathogen attack) and abiotic (for example, heat, cold, salt and drought) stresses (Day *et al.*, 2002). These signals can elicit an increase in the cytosolic calcium concentration, which then initiates downstream signal transduction pathways via Ca²⁺-binding proteins (Dodd *et al.*, 2010). Calmodulin (CaM) is one of the best-characterized Ca²⁺-binding proteins and is also thought to be a primary intracellular Ca²⁺ receptor in all eukaryotes (Babu *et al.*, 1988). CaM is strongly conserved across all species (Hoeftlich & Ikura, 2002). The protein is composed of 148 amino acids, which form two globular domains connected by a long flexible helix (Snedden & Fromm, 1998). CaM conveys stimuli signals through alterations in its own structure in response to calcium binding (Snedden & Fromm, 1998; Zielinski, 1998). Previous structural studies on CaM under different conditions have revealed that CaM adopts a 'closed' conformation in the calcium-free state but adopts an 'open' conformation following calcium binding (Feldkamp *et al.*, 2011; Wang *et al.*, 2012). In the 'closed'

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conformation the hydrophobic residues in each lobe are buried and held together, which precludes further substrate binding; however, calcium saturation triggers a significant change in the orientation of the helices in two lobes, and this change eventually leads to an 'open' conformation with exposed hydrophobic clefts (Houdusse *et al.*, 2006; Wang *et al.*, 2012). This structural change enables CaM to induce signal transduction pathways that act downstream of the initial detection of the second messenger Ca^{2+} (Zielinski, 1998). Thus, plants respond to external signals through these CaM-mediated processes and also through cellular events triggered by other signalling pathways (Arazi *et al.*, 1995).

The *Arabidopsis thaliana* BAG (Bcl-2-associated athanogene) family is a multifunctional group of chaperone regulators. These proteins are evolutionarily conserved and share a common region located near the C-terminus termed the BAG domain (BD), which directly interacts with the ATPase domain of Hsp70/Hsc70 (Kabbage & Dickman, 2008). The BAG family comprises seven members in *Arabidopsis*, and many of them have been shown to be involved in the regulation of apoptosis-like processes, such as those that occur during circumstances ranging from pathogen attack to abiotic stresses and development (Doukhanina *et al.*, 2006). According to their specific functional domain organizations, there are two types of *A. thaliana* BAG (AtBAG) proteins (Fig. 1). Of the seven identified *Arabidopsis* BAG proteins, four (AtBAG1–AtBAG4) possess a ubiquitin-like domain (ULD) at the N-terminus of the BAG domain. However, the other three (AtBAG5–AtBAG7) differ in the N-terminal

region, which contains a CaM-binding domain named the IQ motif (Doukhanina *et al.*, 2006). This plant-specific IQ motif has the consensus sequence IQXXXRGXXXR and preferentially binds to CaM in the absence of calcium (Putkey *et al.*, 2003). This novel feature suggests that these BAG proteins may be regulated by CaM and possibly by Ca^{2+} (Doukhanina *et al.*, 2006). Recently, functional analysis showed that several AtBAG proteins play significant roles in various physiological processes. Induction by abiotic stresses, such as cold, drought, high salt and oxidative stress, appears to be a common feature associated with *bag* genes (Doukhanina *et al.*, 2006). AtBAG4 functions during the response to abiotic stresses such as cold, drought and salt (Doukhanina *et al.*, 2006). AtBAG6 participates in the process of cell death through CaM-mediated cellular responses (Kang *et al.*, 2006). Moreover, AtBAG7 acts as a regulator of the maintenance of the unfolded protein response (UPR) and is active in plants during the response to heat and cold stresses (Williams *et al.*, 2010).

Given that the IQ motif is targeted for CaM binding in the absence of calcium, we speculated that AtBAG5 may perform a role in mediating the cross-talk between Ca^{2+} /CaM signalling and the chaperone system in plants. Therefore, elucidating the direct binding behaviour in the AtBAG5–CaM protein complex could help us to better understand the role of AtBAG5 in the Ca^{2+} /CaM signalling pathway and further clarify the mechanism by which the Ca^{2+} /CaM-mediated signalling pathway is integrated into the potential physiological responses of plants to external stimuli. Additionally, our previous studies on the structures of AtBAG1–AtBAG4 may facilitate the structural determination of the AtBAG5–CaM complex because BAG domains are highly conserved among AtBAG family members (Fang *et al.*, 2013).

To clarify the potential molecular mechanism underlying the interaction between AtBAG5 and CaM in the absence of Ca^{2+} , AtBAG5 (amino acids 49–153, containing both the IQ motif and the BAG domain) and CaM were separately expressed and purified and then crystallized together using a buffer condition without Ca^{2+} . The preliminary X-ray diffraction studies of the protein complex are reported. Structure determination will ultimately provide insights into the mode of interaction between AtBAG5 and calmodulin.

2. Materials and methods

2.1. Molecular cloning, protein expression and purification

The DNA sequence encoding AtBAG5 (residues 49–153) was amplified from the cDNA library of *A. thaliana* using the polymerase chain reaction (PCR) and then cloned into the EcoRI and XhoI sites of an in-house-modified version of the pET-32a vector (Novagen), which only contains a 6×His tag and a PreScission protease cleavage site (LEVLFQGP) at the N-terminus. The amplified sequence was confirmed by DNA sequencing (Invitrogen, People's Republic of China).

The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) CodonPlus cells and the cells were incubated at 310 K to an OD_{600} of 0.6 in LB broth medium containing

Figure 1
Domain structure of the *A. thaliana* BAG proteins. The positions of the ubiquitin-like domain (ULD), BAG domain (BD) and CaM-binding motif (IQ) in the *A. thaliana* BAG family members are shown.

50 $\mu\text{g ml}^{-1}$ ampicillin and then induced with 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 298 K and cultured for a further 16–18 h. The cells were harvested by centrifugation at 5000g for 15 min and resuspended in $T_{20}N_{200}I_{10}$ buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM imidazole)

Figure 2
Characterization of the AtBAG5 (49–153)–CaM complex. (a) SDS-PAGE analysis of the final purity of AtBAG5 (49–153) and CaM. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular-mass marker (labelled in kDa); lane 2, purified AtBAG5 (49–153); lane 3, purified CaM in the buffer containing 5 mM EGTA. For this gel, 10 μg AtBAG5 (49–153) and 20 μg CaM were loaded. (b) Gel-filtration profiles of the AtBAG5 (49–153)–CaM complex, CaM alone and AtBAG5 (49–153) alone using the same HiLoad 26/60 Superdex 200 size-exclusion column. (c) SDS-PAGE analysis of the major elution peak corresponding to the AtBAG5 (49–153)–CaM complex eluted from gel filtration. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular-mass marker (labelled in kDa); lane 2, protein samples mixed in a molar ratio of 1:1 as loaded onto the gel-filtration column; lanes 3–10, fraction samples corresponding to the major elution peak of the protein complex.

containing 1 mM phenylmethylsulfonyl fluoride (PMSF) before cell lysis by sonication. All of the following purification steps were performed at 277 K. After the lysate had been centrifuged at 18 000g for 30 min, the supernatant was loaded directly onto a Ni-NTA agarose column (Qiagen) which was equilibrated with $T_{20}N_{200}I_{10}$ buffer. After the Ni-NTA column had been washed with five column volumes of equilibration buffer, the 6 $\times$ His-tagged protein was eluted with buffer $T_{20}N_{200}I_{300}$ (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 300 mM imidazole). The eluted protein was loaded onto a HiPrep 26/10 desalting column (GE Healthcare) for exchange into buffer $T_{20}N_{200}$ (20 mM Tris-HCl pH 7.5, 200 mM NaCl). After digestion with PreScission protease to remove the His tag, the protein mixture was loaded onto a Ni-NTA agarose column pre-equilibrated with buffer $T_{20}N_{200}$ containing 1 mM PMSF to remove a small amount of contaminant protein. The final target protein was loaded onto a HiLoad 26/60 Superdex 200 size-exclusion column (GE Healthcare) and eluted with $T_{20}N_{200}E_1D_1$ buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) at a flow rate of 2.0 ml min $^{-1}$. The column eluate was collected in 4 ml fractions. The elution peak corresponding to AtBAG5 was identified using 15% Coomassie-stained SDS-PAGE and the sample was pooled together and finally concentrated using Centricon (Millipore).

Rat CaM was expressed in bacteria and purified as described previously (Maune *et al.*, 1992; Huber *et al.*, 1996).

Both of the purified proteins had a purity of greater than 95% (Fig. 2a). The AtBAG5 (49–153)–CaM protein complex was formed by mixing the separately purified proteins together in a 1:1 molar ratio and the mixture was then loaded onto a HiLoad 26/60 Superdex 200 size-exclusion column (GE Healthcare) and finally eluted with $T_{20}N_{500}E_5D_1$ gel-filtration buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 5 mM EGTA, 1 mM DTT). The elution peak corresponding to the protein complex was assessed by 15% Coomassie-stained SDS-PAGE (Fig. 2c). Fractions with the appropriate binding ratio were pooled together and finally concentrated to 15 mg ml $^{-1}$ for use in crystallization (the protein concentration was determined using the Pierce BCA Protein Assay Kit; Thermo Scientific). Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

All crystals were grown by sitting-drop vapour diffusion at 277 and 293 K. The AtBAG5 (49–153)–CaM complex was crystallized by mixing 1 μl protein solution (15 mg ml $^{-1}$ in 20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 5 mM EDTA) with 1 μl precipitant solution in 48-well sitting-drop plates. Up to 12 different screening kits from Hampton Research (Index, Index 2, Crystal Screen, Crystal Screen 2, PEGRx, PEGRx 2, SaltRx, SaltRx 2, PEG/Ion and PEG/Ion 2) and Emerald Bio (Wizard 1 and Wizard 2) were applied in the preliminary crystal screen. Initial crystallization conditions were optimized by changing the concentrations of the precipitants and the pH of the buffer and by the use of additives. Finally, diffraction-quality crystals were obtained 3 d later in a

Table 1

Macromolecule-production information for the AtBAG5–CaM complex.

	AtBAG5	CaM
Source organism	<i>A. thaliana</i>	<i>Rattus norvegicus</i>
DNA source	<i>A. thaliana</i> leaf cDNA	<i>R. norvegicus</i> cDNA
Forward primer	5'-CCGAATTCAACGCCACGCCGCGCAGCA-3'	5'-CCGGATCCATGGCTGACCAACTGACTGAAGAGCAG-3'
Reverse primer	5'-CCCTCGAGCTAGTCTTTAGTCTCCGAGATTGAATCGAG-3'	5'-CCCTCGAGCTATCATTTTGCAGTCATCATTTGTACAAAC-3'
Cloning vector	pEasy Blunt simple cloning vector	pEasy Blunt simple cloning vector
Expression vector	Modified pET-32a (contains only the N-terminal His ₆ tag and a PreScission protease cleavage site)	pET-33b
Expression host	<i>E. coli</i> BL21(DE3) CodonPlus	<i>E. coli</i> BL21(DE3) CodonPlus
Complete amino-acid sequence of the construct produced	GPEFNATAAAARIQSGYRSYRIRNLYKKISSINREANRVQSIIQRQETV-DAIRSDEKERLRMNETLMLLLKLDVPLDPTIREARRKVSRIKIVG-MQEILDSISETKD	GSMADQLTEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTAE-ELQDMINEVDADNGNTIDFPEFLTMMARKMKDTSDEEIEAEFRVFD-KDNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGGQVNYEE-FVQMNTAK

Table 2

Crystallization condition for the AtBAG5–CaM complex.

Method	Sitting-drop vapour diffusion
Plate type	48-well sitting-drop plates
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	15
Buffer composition of protein solution	20 mM Tris–HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 5 mM EDTA
Composition of reservoir solution	2.5 M ammonium sulfate, 0.1 M Tris–HCl pH 8.5
Volume (μl) and ratio of drop	2 μl in total, 1:1
Volume of reservoir (μl)	100

Table 3

Data collection and processing for the AtBAG5–CaM complex.

Values in parentheses are for the outer shell.

Diffraction source	BL-17U1, SSRF
Wavelength (Å)	0.9795
Temperature (K)	100
Detector	MAR 225 CCD
Rotation range per image (°)	0.5
Total rotation range (°)	180
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 64.56, <i>b</i> = 74.89, <i>c</i> = 117.09, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.4335
Resolution range (Å)	50–2.5
No. of unique reflections	19469
Completeness (%)	97.0 (100)
Multiplicity	6.1 (7.3)
$\langle I/\sigma(I) \rangle$	24.6 (6.75)
<i>R</i> _{meas} [†] (%)	18.9 (78.7)

[†] $R_{\text{meas}} = \frac{\sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where *N(hkl)* is the multiplicity, *I_i(hkl)* is the intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is the average value over multiple measurements.

condition consisting of 2.5 M ammonium sulfate, 0.1 M Tris–HCl pH 8.5 at 293 K (Fig. 3*a*). Crystals were recovered in 0.2–0.3 mm nylon loops (Hampton Research) and immediately flash-cooled in liquid nitrogen using Paratone-N as a cryo-protectant. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

X-ray data were collected on beamline BL-17U1 at the Shanghai Synchrotron Radiation Facility (SSRF). Diffraction experiments were conducted at 100 K and the images were

recorded on a 225 mm MAR CCD camera (MAR Research, Norderstedt, Germany). Data were processed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997). The data-collection and processing statistics are summarized in Table 3.

(a)

(b)

Figure 3

Characterization of the crystals of the complex. (a) Crystals of the AtBAG5 (49–153)–CaM complex. (b) SDS–PAGE analysis of the dissolved crystals of the AtBAG5 (49–153)–CaM complex. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular-mass marker (labelled in kDa); lane 2, dissolved crystals of the complex.

3. Results and discussion

3.1. Preparation and confirmation of the protein complex

Both AtBAG5 (49–153) and CaM were highly over-produced in *E. coli* BL21(DE3) CodonPlus cells and were purified following the protocols described in §2. The final purity was confirmed by SDS–PAGE (Fig. 2*a*). The calculated molecular weights of AtBAG5 (residues 49–153) and CaM are 12 and 16.8 kDa, respectively. The main elution peak of the AtBAG5 (49–153)–CaM mixture from the HiLoad 26/60 Superdex 200 size-exclusion column corresponded to a molecular weight of approximately 30 kDa, which differed from that of either AtBAG5 or CaM when eluted alone using gel filtration (Fig. 2*b*). Analysis of the elution peak on 15% SDS–PAGE revealed two bands with molecular weights of 12 kDa for AtBAG5 (49–153) and 16 kDa for CaM (Fig. 2*c*), confirming that the protein complex had formed. Combining the results together, we conclude that the mixture of AtBAG5 (49–153) and CaM is eluted from gel filtration as a protein complex with a molar binding ratio of 1:1.

3.2. Crystallization

Crystals of the protein complex were obtained after 3 d by the sitting-drop vapour-diffusion technique at 293 K using SaltRx (Hampton Research, California, USA) condition F3 (0.1 M Tris–HCl pH 8.5, 1.5 M ammonium sulfate; Fig. 3*a*). Further crystallization optimization experiments were performed by changing the salt (precipitant) concentration and the buffer pH. Eventually, we obtained well shaped larger

crystals suitable for X-ray analysis in a condition consisting of 0.1 M Tris pH 8.5, 2.5 M ammonium sulfate. Crystals were also picked up, washed in the crystallization solution three times prior to dissolution in the gel-filtration buffer and finally loaded onto a 15% SDS–PAGE gel to confirm that they were formed of the protein complex (Fig. 3*b*). Diffraction-quality crystals were flash-cooled in liquid nitrogen using Paratone-N as a cryoprotectant.

3.3. Data collection and processing

A complete native diffraction data set was collected and processed to a resolution of 2.5 Å on beamline BL-17U1 at SSRF. One of the diffraction patterns is shown in Fig. 4. The data were processed and scaled using the *HKL-2000* (Otwinowski & Minor, 1997) and *CCP4* (Winn *et al.*, 2011) program suites. The crystals of the AtBAG5 (49–153)–CaM complex belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 64.56$, $b = 74.89$, $c = 117.09$ Å. Resolution-dependent Matthews coefficient probability analysis suggested the presence of two molecules per asymmetric unit, with a solvent content of approximately 50% and a V_M value of $2.44 \text{ Å}^3 \text{ Da}^{-1}$. The data-processing statistics are given in Table 3.

The crystallization and preliminary X-ray crystallographic studies of the AtBAG5 (49–153)–CaM complex provide the possibility of solving its structure. Molecular replacement using the coordinates of AtBAG4 (PDB entry 4hwh; Fang *et al.*, 2013) as a template was attempted using the *Phaser* molecular-replacement program (McCoy, 2007) as imple-

Figure 4

A representative diffraction image from a crystal of the AtBAG5 (49–153)–CaM complex. The inset shows weak spots at the edge corresponding to a resolution of 2.85 Å. A complete data set was collected to a resolution of 2.5 Å.

mented in the CCP4 suite (Winn *et al.*, 2011) when searching for AtBAG5 molecules. Moreover, attempts to use a selenomethionine derivative of CaM to replace the wild-type CaM in the AtBAG5 (49–153)–CaM complex for MAD/SAD phasing are in progress. The selenomethionines in CaM could be used as a guide to solve the phase problem of the CaM molecules in the complex.

Further structural determination of the AtBAG5–CaM complex will provide a detailed description and structural insights into the interaction between the IQ motif and CaM in the absence of Ca²⁺ and will further improve the understanding of the role of AtBAG5 in Ca²⁺/CaM signalling in plants.

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