

STRUCTURE NOTE

Crystal structure of the protein At3g01520, a eukaryotic universal stress protein-like protein from *Arabidopsis thaliana* in complex with AMP

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ABSTRACT

Members of the universal stress protein (USP) family are conserved in a phylogenetically diverse range of prokaryotes, fungi, protists, and plants and confer abilities to respond to a wide range of environmental stresses. *Arabidopsis thaliana* contains 44 USP domain-containing proteins, and USP domain is found either in a small protein with unknown physiological function or in an N-terminal portion of a multi-domain protein, usually a protein kinase. Here, we report the first crystal structure of a eukaryotic USP-like protein encoded from the gene At3g01520. The crystal structure of the protein At3g01520 was determined by the single-wavelength anomalous dispersion method and refined to an *R* factor of 21.8% (*R*_{free} = 26.1%) at 2.5 Å resolution. The crystal structure includes three At3g01520 protein dimers with one AMP molecule bound to each protomer, comprising a Rossmann-like α/β overall fold. The bound AMP and conservation of residues in the ATP-binding loop suggest that the protein At3g01520 also belongs to the ATP-binding USP subfamily members.

Proteins 2015; 83:1368–1373.

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Key words: universal stress protein; *Arabidopsis thaliana*; At3g01520.

Grant sponsor: National Institute of Health; grant numbers: U01 GM098248, U54 GM074901, and P50 GM64598 (Protein Structure Initiative); Grant sponsor: Ministry of Science, ICT and Future Planning of Korea; grant number: 2011-0030001 and 2013M3A6A4043695 (Tumor Microenvironment Global Core Research Center and Global Frontier Project funded through the National Research Foundation); Grant sponsor: Offices of Biological and Environmental Research; Grant sponsor: Basic Energy Sciences of the US Department of Energy; Grant sponsor: National Center for Research Resources of the National Institutes of Health.

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Received 14 January 2015; Revised 20 April 2015; Accepted 24 April 2015

Published online 28 April 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/prot.24821

INTRODUCTION

The gene At3g01520 of *Arabidopsis thaliana* encodes 175-residue universal stress protein (USP)-like protein (Pfam accession: PF00582), which is widely found in the genomes of bacteria, as well as fungi, protozoa, and plants.¹ The USP superfamily represents a set of small cytoplasmic proteins whose expressions are affected by a wide range of internal or external stresses.^{1,2} Genetic evidence has subsequently shown that USP mediates survival of cells in response to a wide variety of stress states, including nutrient starvation, exposure to heat, acid, heavy metals, oxidative agents, osmotic stress, antibiotics, and uncouplers of oxidative phosphorylation.^{3–5}

Structures of many bacterial and viral USP proteins have been solved so far. Interestingly, while the USP domain structure of MJ0577 from *Methanocaldococcus jannaschii* was solved with a bound ATP, *Haemophilus influenzae* UspA lacks both ATP-binding activity and ATP-binding residues.^{6,7} This suggests that the USP domain family proteins fall into two major groups depending on their nucleotide binding capabilities. However, despite the knowledge of bacterial USP proteins, the functional diversity of the USPs in other organisms, including various plant species, has not been well defined.⁸

A. thaliana contains 44 USP domain-containing proteins, and USP domain is found either in a small protein with unknown physiological function or in an N-terminal portion of a multidomain protein, usually a protein kinase.¹ Most of the *A. thaliana* USP-like proteins are annotated as “adenine nucleotide α -hydrolase-like superfamily protein”, although it has not been reported whether they bind or hydrolyze ATP.^{9,10} In this study, we report the crystal structure of a eukaryotic USP-like protein At3g01520 from *A. thaliana* in complex with AMP at 2.5 Å resolution. This work represents the first structure of eukaryotic USP family protein, thus contributing to expand our knowledge on the structural features of the diverse USP protein family members.

MATERIALS AND METHODS

Native and SeMet-labeled At3g01520 proteins were cloned and purified following the standard Center for Eukaryotic Structural Genomics (CESG) pipeline protocol described in detail elsewhere.^{11–14} Crystals of the protein At3g01520 were grown at 293 K by the hanging-drop method from 10 mg mL⁻¹ protein solution in buffer (50 mM NaCl, 3 mM NaN₃, 0.3 mM TCEP, and 5 mM MES-NaOH, pH 7.0) mixed with an equal amount of well solutions containing 18% (v/v) PEG 2K, 5% (v/v) DMSO, and 100 mM PIPES, pH 6.5. They belong to space group *P1*, with unit-cell parameters $a = 63.35$, $b = 65.66$, $c = 73.01$ Å, $\alpha = 75.45$, $\beta = 75.04$, $\gamma = 66.11^\circ$. Crystals were cryoprotected by soaking in a

cryosolution containing 20% (v/v) PEG 2K and 100 mM Tris-HCl, pH 8.0, and a final concentration of 20% (v/v) ethylene glycol. X-ray diffraction data for native and selenomethionine crystals were collected at the 22-ID synchrotron beamlines at the Advanced Photon Source of Argonne National Laboratory.

We have solved the crystal structure of At3g01520 by SAD phasing at 2.50 Å resolution. Twenty selenomethionine sites were located with the program SHELXD.¹⁵ After initial refinement, six At3g01520 protomers were defined in an asymmetric unit. Out of 18 methionine residues in an asymmetric unit, two of the residues showed definable alternate conformations, explaining the two extra selenomethionine sites found with SHELXD. The selenomethionine sites were used to calculate the phases with RESOLVE.¹⁶ The SAD-phased electron density map was of high quality and was readily interpreted by the automatic model building procedure of RESOLVE.¹⁶ The structure was completed using alternate cycles of manual building in Xfit¹⁷ and refinement in REFMAC.¹⁸ Noncrystallographic symmetry constraints, as defined in the standard refinement protocol of Refmac,¹⁸ were applied throughout the refinement. NCS constraints were not released due to low redundancy of the data as the structure was refined in *P1* space group. All refinement steps were monitored using an R_{free} value based on 5.3% of the independent reflections. The stereochemical quality of the final model was assessed using PROCHECK¹⁹ and MolProbity.²⁰ The coordinates and structure factors have been deposited in the Protein Data Bank (<http://www.pdb.org>) for immediate release with PDB ID code 2GM3. Table I summarizes the data collection, phasing, and model refinement statistics.

Analytical size-exclusion chromatography was performed on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 50 mM Bis-Tris at pH 7.0, 150 mM NaCl, 0.3 mM TCEP at 277 K. The samples were injected using a 0.25 mL loop, and the flow rate was 0.5 mL/min for 36 mL. Six protein standards from Gel Filtration Marker Kit (Sigma-Aldrich) were used to calibrate the column. The protein standards used were thyroglobulin (MW 669,000), apoferritin (MW 443,000), β -amylase (MW 200,000), alcohol dehydrogenase (MW 150,000), bovine serum albumin (MW 66,000), and carbonic anhydrase (MW 29,000). Output data were subsequently exported and analyzed in Excel (Microsoft).

RESULTS AND DISCUSSION

We have determined the crystal structure of the protein At3g01520 in complex with AMP. The final model includes six AMP-bound protomers in a crystallographic asymmetric unit. The R and R_{free} factors were 22.1 and 26.1%,

Table 1

Statistics for Data Collection, Phasing, and Model Refinement

Data collection and phasing ^a	
Space group	P1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	63.35, 65.66, 73.01
α , β , γ (°)	75.45, 75.04, 66.11
Data set	Se λ 1 (peak)
X-ray wavelength (Å)	0.9793
Resolution (Å) ^b	50.00–2.50 (2.59–2.50)
Total/unique reflections	133,684/36,006
Completeness (%)	97.2 (89.4)
<i>R</i> _{merge} (%) ^c	5.8 (23.9)
Figure of merit ^d for SAD phasing: 0.51	
Refinement	Data set: Se λ 1 (peak)
Resolution (Å)	50.0–2.50
<i>R</i> _{work} / <i>R</i> _{free} ^e	0.221/0.261
No. of protein atoms/mean B-factor (Å ²)	7,084 ^g /60.5
No. of ligand atoms (AMP)/mean B-factor (Å ²)	138/47.1
No. of solvent atoms/mean B-factor (Å ²)	13/46.1
Ramachandran plot analysis (for Chain A)	
Most favored regions	143 (97.3%)
Additional allowed regions	4 (2.7%)
Disallowed regions	0 (0%)
R.m.s. deviations from ideal geometry	
Bond lengths (Å)	0.016
Bond angles (°)	1.50

^aData collected at the Sector 22-ID of the Advanced Photon Source.^bNumbers in parentheses indicate the highest resolution shell of 20.^c $R_{\text{merge}} = \sum_h \sum_i |I(h)_i - \langle I(h) \rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the observed intensity of reflection h , and $\langle I(h) \rangle$ is the average intensity obtained from multiple measurements.^dFigure of merit = $\langle \sum P(\alpha) e^{i\alpha} / \sum P(\alpha) \rangle$, where α is the phase angle and $P(\alpha)$ is the phase probability distribution.^e $R_{\text{work}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where $|F_o|$ is the observed structure factor amplitude and $|F_c|$ is the calculated structure factor amplitude.^f $R_{\text{free}} = R$ -factor based on 5.1% of the data excluded from refinement.^gNumber of nonhydrogen protein atoms included in refinement.

respectively, for the 20.00–2.50 Å data (Table 1). The structure is resolved from residue 5 to residue 175. Loops comprising residues 56–65 and 137–145 are missing in all the chains in the asymmetric unit, suggesting that these are highly flexible regions. One At3g01520 protomer comprises a central five-stranded, parallel β -sheet (β 4– β 5– β 1– β 2– β 3), which is surrounded by five α -helices [Fig. 1(A)]. It is folded into the classic Rossmann-like α/β -fold, resembling the structures of other reported structures of USP family members.^{6,7,21–24}

Although it was previously reported that the protein At3g01520 lacks dimer-forming hydrophobic β 5 region,⁹ the hydrophobic β 5 sequence is highly conserved and the protein At3g01520 also exists as a dimer in the crystal [Fig. 1(B,C)]. The protein At3g01520 has buried surface area of 1,017 Å² per monomer, which corresponds to ~12% of the monomer surface area (87,875 Å²). The central β -sheets of the two protomers form an extended β -sheet by β 5 strands from each protomer in the dimer structure and this feature has been widely observed in other reported USP structures in a dimeric state [Fig. 1(B)].^{6,7,21–24} The dimer interface is mediated through van der Waals packing of a number of hydrophobic resi-

dues (Ile38, Phe46, Phe127, Pro157, and Met159) as well as electrostatic interactions formed by side chains of Thr6, Arg40, Asn42, Asp45, and Asp126. Based on the dimeric interface analysis from crystallographic dimer packing and size-exclusion chromatography data [Fig. 1(D)], we propose that the protein At3g01520 forms a dimer for its physiological conditions.

The USP family proteins fall into two groups depending on their ATP-binding capabilities.^{7,9} In the case of the protein At3g01520, there have been mixed predictions for its ATP-binding capability. According to the overall sequence similarity of At3g01520 to MJ0577 (Protein Data Bank, PDB, ID 1 MJH), which contained naturally acquired ATP in its solved crystal structure, the protein At3g01520 has been classified as a 1 MJH-like plant protein. However, extensive sequence analysis could not reveal conserved ATP-binding residues such as Asp and Ser that have been predicted as ATP-binding residues from the crystal structure of 1 MJH.⁹ Interestingly, after model building of all the protein residues in a crystallographic asymmetric unit, the *F_o – F_c* omit electron density map clearly showed positive extra electron densities around the similar ATP-binding site of MJ0577 in all six protomers in the asymmetric unit, which could be definitely modeled as an AMP molecule [Figs. 1(A,B) and 2(A)]. Since we did not add any nucleotide supplements in culture media for *E. coli*, this naturally acquired AMP molecule from *E. coli* could be reminiscent of ATP molecule of other ATP-binding USP proteins. Thus, this observation strongly supports that the protein At3g01520 also belongs to the nucleotide-binding USP subfamily members.

Whether the preferred ligand of the protein At3g01520 is ATP or AMP has not been known. However, key residues for the AMP interaction exist on a flexible loop that corresponds to the ATP-binding sites of homologous USP proteins. The bound AMP molecule is buried within a cleft near the surface mainly interacting with a number of main chain nitrogen and carbonyl oxygen atoms [Figs. 1(A) and 2(A)], resembling other AMP/ATP-bound USP structures.^{6,21,23,24} The ATP-binding USP subfamily members adopts a similar conformation of ATP-binding loops with a consensus motif G-2x-G-9x-G-(S/T) (x stands for any amino acid).^{7,21–24} This ATP-binding consensus sequence motif is also strictly conserved in At3g01520 [Fig. 1(C)]. The phosphate group of the bound AMP is exposed to the solvent, leaving enough room for β - and γ -phosphates for ATP binding. However, unlike other ATP-bound USP structures, the conserved ATP-binding loop region of the protein At3g01520 could not be fully defined in our crystal structure and it may be caused by the absence of β - and γ -phosphate moiety in the case of the bound AMP, resulting in a degree of conformational flexibility. Presumably, the disordered loop of the protein At3g01520 may be able to form an optimum structure in the event

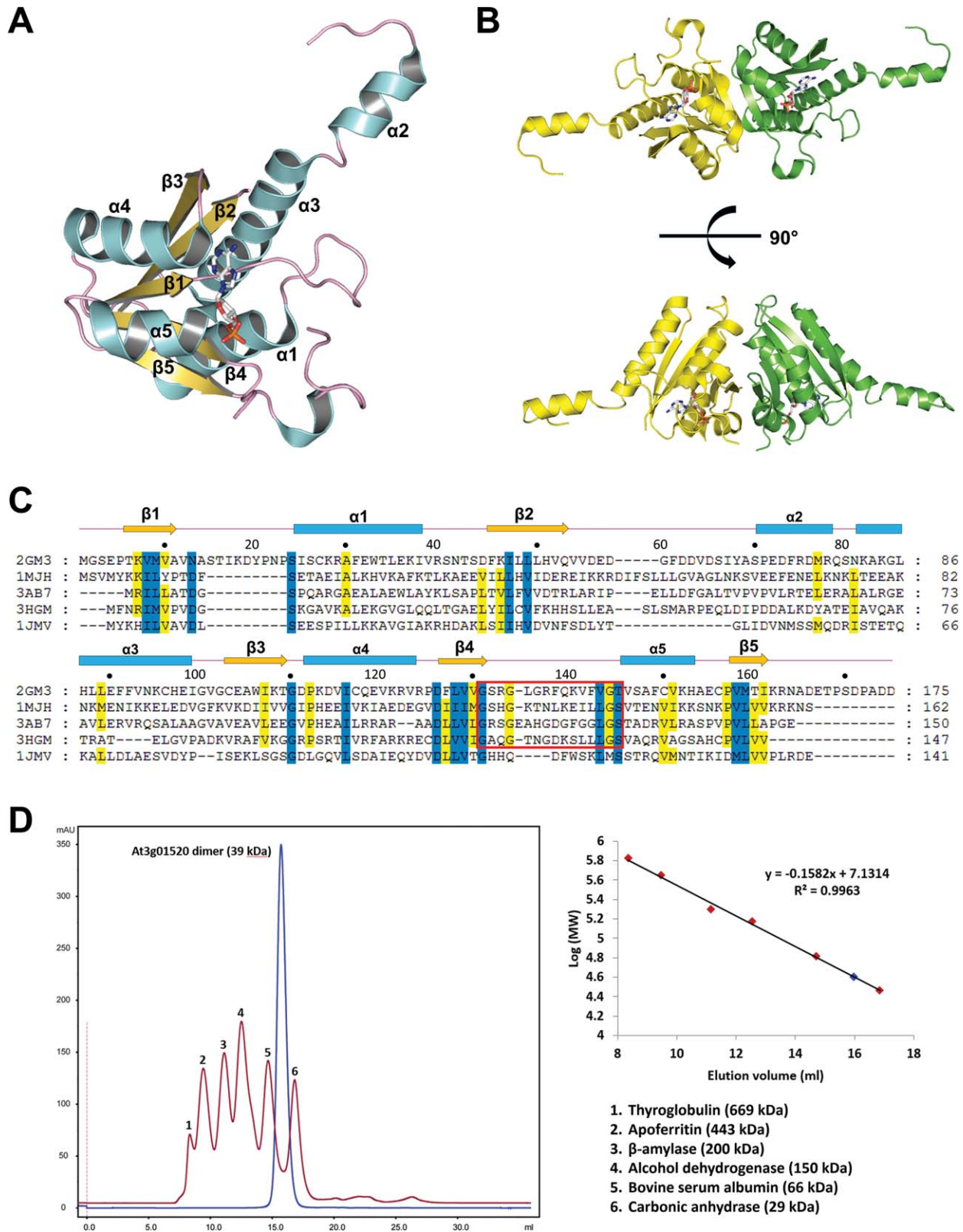
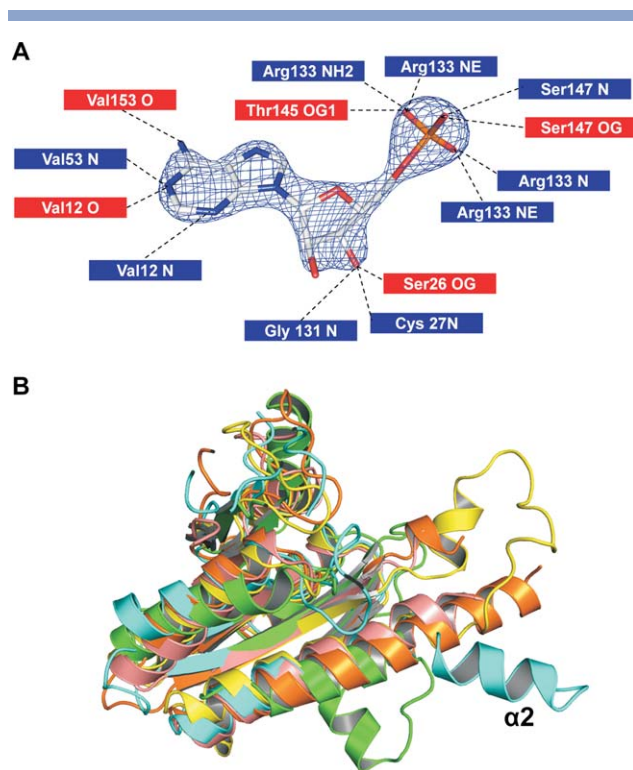


Figure 1

Overall structure of the protein At3g01520. **A:** Monomer structure of the protein At3g01520. α -helices, β -strands, and loops are colored in cyan, yellow, and pink, respectively. The bound AMP is shown in a stick model. **B:** Dimerization of the protein At3g01520. Chain A and B are colored in yellow and green, respectively. The bound AMP is shown in a stick model. **C:** Multiple sequence alignment of USP proteins from *A. thaliana* (PDB ID: 2GM3), *M. jannaschii* (PDB ID: 1 MJH), *T. thermophilus* (PDB ID: 3AB7), *H. elongata* (PDB ID: 3HGM), and *H. influenzae* (PDB ID: 1JMV). The conserved G-2X-G-9X-(S/T) motif is boxed in red. α -helices and β -strands are indicated above the sequences as cyan cylinders and yellow arrows, respectively. **D:** Analytical size-exclusion chromatograms of At3g01520 (blue line) and size markers (magenta line). The position of molecular weight markers is indicated for comparison.

**Figure 2**

Interaction of the protein At3g01520 with bound AMP. **A:** *Fo*-*Fc* electron density map of the bound AMP contoured at 3.0 σ . **B:** Aligned monomeric structures of USP proteins. USP proteins from *A. thaliana*, *M. jannaschii*, *T. thermophilus*, *H. elongate*, and *H. influenzae* are colored in cyan, orange, pink, yellow, and green, respectively. The $\alpha 2$ helix of the protein At3g01520 is labeled below.

of interaction with a proper ligand, such as ATP, by reorienting and ordering the flexible loop.

A structural homology search was conducted using the DALI server.²⁵ Close structural homologs of the protein At3g01520 (DALI Z score higher than 15) include a number of established ATP-binding USP subfamily proteins: USP domain of MJ0577 from *M. jannaschii* with Z score 15.7, r.m.s.d. distance 3.5 Å [mt]128 aligned C_{α} residues, and 20% sequence identity (PDB ID 1 MJH⁶); N-terminal USP domain of tandem-type USP TTHA0350 from *Thermus thermophilus* HB8 with Z score 15.5, r.m.s.d. distance 2.4 Å over 118 aligned C_{α} residues, and 20% sequence identity (PDB ID 3AB7²⁴); USP TeaD from *Halomonas elongata* with Z score 15.3, r.m.s.d. distance 3.9 Å over 120 aligned C_{α} residues, and 23% identity (PDB ID 3HGM²¹). UspA from *H. influenzae* also shares a high level of structural similarity with Z score 15.7, r.m.s.d. distance 3.5 Å over 128 aligned C_{α} positions, and 20% sequence identity (PDB ID 1JMV⁷), despite its lack of ATP-binding capability and altered ATP-binding loop sequence [Fig. 1(C)]. Interestingly, even though these

USP subfamily members share a high degree of structural similarities, the region corresponding to $\alpha 2$ (residues 70–78) of the protein At3g01520 structure shows distinctive conformational diversity [Fig. 2(B)]. It is plausible to speculate that the region is involved in diverse interactions with other cellular components in response to stress signals in various pathways.

In conclusion, we report the first crystal structure of a eukaryotic USP family protein. Its structural similarity with other ATP-binding USP subfamily proteins, as well as the bound AMP and conservation of residues in the ATP-binding loop, suggest that the protein At3g01520 also belongs to the ATP-binding USP subfamily members. The biological function of the protein At3g01520 still remains elusive. However, it has been previously suggested that ATP-binding USP proteins might function as a molecular switch much like the Ras protein family, whose GTP hydrolysis ability is modulated by interaction with a number of regulatory proteins.²² It is therefore plausible that At3g01520 possibly functions as a molecular switch by its putative ATP-binding and ATP-hydrolyzing properties. Further structure-guided biochemical studies to investigate how potential ATP-hydrolyzing activity of the protein At3g01520 is related to physiological phenotypes will shed light on the role of this uncharacterized USP protein in a wide range of stress-related signaling pathways in *A. thaliana*.

ACKNOWLEDGMENTS

Special thanks go to all the members of CESG team. Data for this study were collected at the 22-ID beamline at the Advanced Photon Source (APS) of Argonne National Laboratory

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