Structural and Functional Assays of AtTLP18.3 Identify Its Novel Acid Phosphatase Activity in Thylakoid Lumen

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Abbreviations: AtTLP18.3: Arabidopsis thaliana thylakoid lumen protein of 18.3 kDa; cTP: chloroplast transit peptide; DUF477: domain with unknown function 477; TMH: transmembrane helix; PS: photosystem; Cyt: cytochrome; TAP: thylakoid acid phosphatase; pNPP: p-nitrophenyl phosphate; DiFMUP, 6,8 difluoro-4-methylumbelliferyl phosphate; pSer: O-phospho-L-serine; RMSD, root mean square deviation.
ABSTRACT

The membrane protein AtTLP18.3 contains a domain of unknown function, DUF477; it forms a polysome with photosynthetic apparatuses in the thylakoid lumen. To explore the molecular function of AtTLP18.3, we resolved its crystal structures with residues 83 to 260, the DUF477 only, and performed a series of biochemical analyses to discover its function. The gene expression of AtTLP18.3 followed a circadian rhythm. X-ray crystallography revealed the folding of AtTLP18.3 as a three-layer sandwich with 3 α-helices in the upper layer, 4 β-sheets in the middle layer, and 2 α-helices in the lower layer, which resembles a Rossmann fold. Structural comparison suggested that AtTLP18.3 might be a phosphatase. The enzymatic activity of AtTLP18.3 was further confirmed by phosphatase assay with various substrates (e.g., p-nitrophenyl phosphate, 6,8 difluoro-4-methylumbelliferyl phosphate, O-phospho-L-Serine [pSer] and several synthetic phosphopeptides). Furthermore, we obtained the structure of AtTLP18.3 in complex with pSer to identify the binding site of AtTLP18.3. Our structural and biochemical studies revealed that AtTLP18.3 has a molecular function of a novel acid phosphatase in the thylakoid lumen. DUF477 is accordingly renamed the thylakoid acid phosphatase (TAP) domain.
INTRODUCTION

Chloroplasts in higher plants are the center of photosynthesis. In recent years, proteomic studies of the chloroplastic envelope membrane (Ferro et al., 2003; Froehlich et al., 2003), thylakoid membrane (Friso et al., 2004; Peltier et al., 2004), and luminal fraction (Peltier et al., 2002; Schubert et al., 2002; Goulas et al., 2006) have provided clearer knowledge of the chloroplastic proteome of Arabidopsis thaliana. The most abundant protein complexes, such as photosystem (PS) II and I, cytochrome (Cyt) b6f, and ATP synthase, are photosynthetic apparatuses located at the thylakoid membrane for biogenesis. Intensive research into the photosynthetic protein complexes resolved the crystal structures of PSII/I, Cyt b6f and ATP synthase (Jordan et al., 2001; Zouni et al., 2001; Groth, 2002; Stroebel et al., 2003; Loll et al., 2005; Amunts et al., 2007; Umena et al., 2011). Combined with biochemical and functional analysis, structural studies have provided the detailed orientation of the cofactors and the molecular mechanism for photosynthesis. However, a number of auxiliary proteins, including protein translocons, kinases, phosphatases, proteases, chaperones and many proteins with unknown function, have been isolated by proteome analyses. The proportion of proteins with unknown function is about 25% for the 384 thylakoid proteins (Peltier et al., 2004). The unknown auxiliary proteins are challenging targets because of their low abundance or transient expression at a particular stage (Klimmek et al., 2006). An alternative approach to exploring a molecular function for an unknown protein is to resolve its 3-D structure (Hwang et al., 1999; Lee et al., 2001).

The AtTLP18.3 protein located at the thylakoid lumen is 18.3 kDa (Peltier et al., 2002; Schubert et al., 2002; Friso et al., 2004). On the basis of bioinformatics analysis, the AtTLP18.3 protein can be divided into 3 regions: a transit peptide for importing into the chloroplast and thylakoid lumen, residues 1 to 82; a domain of unknown function 477 (DUF477), residues 83 to 235; and a potential transmembrane helix (TMH), residues 260 to 282. The mature form of AtTLP18.3 consists of DUF477 and TMH for anchoring at the thylakoid membrane. In previous study, the knockout mutant of AtTLP18.3 did not show an observable phenotype under normal growth conditions but showed retarded
growth under fluctuating light (Sirpio et al., 2007; Mulo et al., 2008). The protein was proposed to provide partial protection for the PSII complex to resist photoinhibition under fluctuating high-light conditions (Sirpio et al., 2007). Furthermore, except for research involving microarrays and proteomics, which has alluded to this gene, no research has focused on the function of this gene. Investigating the molecular function of AtTLP18.3 will provide valuable insights into its role in the thylakoid lumen.

DUF477 represents a superfamily of uncharacterized proteins and can be found in eukarya and eubacteria (Finn et al., 2010). We aimed to resolve the 3-D structure of AtTLP18.3 to explore its molecular function by structural comparison and functional characterization. We prepared and crystallized a truncated AtTLP18.3 from residues 83 to 260, without the TMH, then structurally compared the resolved structure of AtTLP18.3 and showed a folding similar to that of phosphatases. The phosphatase activity of AtTLP18.3 was further analyzed. In addition, the structure of AtTLP18.3 in complex with O-phospho-L-serine (pSer) was resolved to confirm its substrate binding site and acid phosphatase activity. We discuss the possible role of AtTLP18.3 in photosystem repair in chloroplasts.
RESULTS

Transcripts of AtTLPI8.3 followed a diurnal rhythm

We analyzed the promoter region 1500 bp upstream of the start codon by using both the PLACE (Higo et al., 1998; Higo et al., 1999) and PlantCARE (Rombauts et al., 1999; Lescot et al., 2002) software. As shown in Figure 1A, the existence of cis-acting regulatory elements of a sugar-repressive element (S), a phytochrome-regulation element (P), a CCA1-binding site (C), and a gibberellin (GA)-responsive element (G) implied that AtTLPI8.3 might be controlled by the circadian rhythm, sucrose, and gibberellin. We compared the diurnal expression patterns of AtTLPI8.3 in circadian clock associated 1 (CCA1)-overexpressing (CCA1-ox) mutants and wild-type Arabidopsis. Northern blot analysis revealed AtTLPI8.3 with a diurnally fluctuating expression pattern in wild-type plants; the transcripts showed 2 high expression peaks, at 8 and 16 h after lights were turned on (Figure 1B and 1C). In the CCA1-ox mutant, the diurnal rhythmic expression pattern was still observable but slightly disrupted (Figure 1C and 1D).

The protein AtTLPI8.3 with DUF477 is conserved from cyanobacteria and algae to higher plants

We used the amino acid sequence of AtTLPI8.3 to search for homologs with the NCBI blastp program against the non-redundant protein sequence database (Altschul et al., 1997). No homologs could be found in animal cells. The protein AtTLPI8.3 is conserved from cyanobacteria and algae to green plants and is found in photosynthetic organisms. AtTLPI8.3 belongs to an orthologous group specific to algae and green plants. The N terminus of AtTLPI8.3 (residues 1 to 82) was predicted as the transit peptide for importing into the chloroplast and thylakoid lumen (Figure 2A). The mature form of AtTLPI8.3 (residues 83 to 285) comprises DUF477 (residues 83 to 235) and the TMH (residues 260 to 282). Amino acid sequence alignment revealed the mature form of the AtTLPI8.3 protein with 83% identity and 96% similarity to the homolog of Oryza sativa (japonica cultivar-group), 66% identity and 86% similarity to that of Selaginella tamariscina, 47% identity and 66% similarity to that of Ostreococcus lucimarinus.
CCE9901, 32% identity and 51% similarity to that of *Synechocystis* sp. PCC 6803, and 31% identity and 52% similarity to that of *Cyanothece* sp. PCC 7424 (Figure 2A).

**Structure determination and overall structure of AtTLP18.3 reveal its α/β folds**

Because the phenotype of AtTLP18.3 could not be observed by knockout or overexpression experiments under normal growth conditions (Sirpio et al., 2007), investigating the molecular structure suggested an alternative approach to explore its function. Therefore, we aimed to resolve the structure of AtTLP18.3 by x-ray crystallography and propose its molecular function by comparing the structure with the known function of other proteins (Lee et al., 2001). We constructed the truncated AtTLP18.3 from residues 83 to 260 (Figure 2B). Because AtTLP18.3 lacks a methionine residue, we introduced 5 methionine residues into AtTLP18.3 by site-directed mutagenesis (Supplemental Table S1 online) (i.e., L107M, L128M, I133M, I159M, and L202M) for solving the phase by x-ray crystallography. The selected point mutations were located near the loop region by predicting the secondary structure with the Psipred server (McGuffin et al., 2000). After a screening, we could obtain only the crystals of the protein with double mutation, L128M and I159M (TLP_M45; as shown in Figure 2A).

The crystal of TLP_M45 belongs to an orthorhombic space group P2_12_1, with unit-cell parameters a=46.9 Å, b=49.8 Å, c=76.7 Å, α=β=γ=90° and diffraction to a resolution of 2.6 Å. Two selenomethionine residues were found after a search by use of SOLVE/RESOLVE (Terwilliger and Berendzen, 1999) with the method of peak wavelength in single-wavelength anomalous dispersion (SAD). After model rebuilding and refinement, the final structure of TLP_M45 reached 20.1% R-factor and 26.8% R_free. Because the crystals of native AtTLP18.3 were isomorphous to TLP_M45, the structure could be adapted from the structure of TLP_M45. The resolution of native AtTLP18.3 was further diffracted to 1.6 Å, and the structure of AtTLP18.3 was refined to 20.3% R-factor and 22.6% R_free. All crystallography statistics are in Table 1.

The final structure of truncated AtTLP18.3 contained only a domain with a globular shape from residues 83 to 235 and the C-terminal region from residues 236 to 260, which had no electron density for model building. The domain showed 8 α-helices and 4 β-sheets. The domain architecture belongs to an α/β folding similar to a “hamburger” shape.
(Figure 3A). The lower layer consists of 2 helices by \( \alpha_1 \) from Ser83 to Asp92, and \( \alpha_3 \) from Asp143 to Tyr156. The upper layer consists of 3 \( \alpha \)-helices: (1) \( \alpha_2 \) from Ser108 to Lys127, (2) \( \alpha_6 \) from Glu191 to Asp208, and (3) \( \alpha_7 \) from Lys210 to Asp227. The middle layer is formed by 4-stranded \( \beta \)-sheets with (1) \( \beta_1 \) from Tyr99 to Asp103, (2) \( \beta_2 \) from Arg129 to Val135, (3) \( \beta_3 \) from Asn164 to Thr172 and (4) \( \beta_4 \) from Glu176 to Gly181. Two short \( \alpha \)-helices, \( \alpha_4 \) from Ser158 to Asn163 and \( \alpha_5 \) from Gly182 to Gly190, are perpendicular to the plane of the middle layer and provide the connection for overall structure of the domain (Figure 3A). We calculated the molecular surface with electrostatic potential (Figure 3C). In the left panel of Figure 3C, this side was proposed to be the active site in the center with the positive charge surrounding the negative charge. By rotating the molecular surface 180° (in the right panel of Figure 3C), this side showed almost the entire surface with a negative charge.

Structure comparison suggests that AtTLP18.3 functions as a phosphatase

To investigate the molecular function of AtTLP18.3 from the resolved structure, we submitted the coordinates of AtTLP18.3 to the CATH server (Kawabata, 2003; Greene et al., 2007) to reveal its structure classification. The results indicated that the folding of AtTLP18.3 belongs to a Rossmann fold (CATH code: 3.40.50) with an architecture of a 3-layer (\( \alpha \beta \alpha \)) sandwich. For advanced searching by structure comparison, we submitted the coordinates of AtTLP18.3 to the programs Matras (Kawabata, 2003) and DALI (Holm and Sander, 1998). AtTLP18.3 shared the highest structural similarity in protein size with a mannitol-specific cryptic phosphotransferase enzyme IIA CmtB from *Escherichia coli* (PDB ID: 2OQ3) (Figure 3B). The Z-score between AtTLP18.3 and CmtB was 12.25 and the root mean square deviation 4 Å. These 2 proteins have low sequence identity, 12%. High Z-scores in the Matras list provided more evidence to suggest that AtTLP18.3 might be a phosphatase, such as the exopolyphosphatase-related protein (PDB ID: 3DMA, Z-score = 14.9, sequence identity = 13.2%) and the cytosolic exopolypophosphatase (PDB ID: 2QB7, Z-score = 11, sequence identity = 13.2%). AtTLP18.3 might function as an enzyme that transfers or removes a phosphate group.
from a protein. Therefore, we proposed that AtTLP18.3 might be a phosphatase that
removes the phosphate group from phosphorylated proteins.

Phosphatase activity assay of AtTLP18.3

We first analyzed the phosphatase activity of AtTLP18.3 by use of the general substrate
p-nitrophenyl phosphate (pNPP), a non-proteinaceous and non-specific substrate for acid
and alkaline phosphatase assay. Preliminary results indicated that AtTLP18.3 contains
phosphatase activity. Consequently, we further determined the optimal conditions and
enzymatic kinetics of AtTLP18.3 for pNPP. AtTLP18.3 showed the highest activity with
3.5 to 4.5 pH (Figure 4A) and 30 to 45°C (Figure 4B). The pNPP substrate binding
affinity (Km) was 42.56 mM and the catalytic velocity (Vmax) 8.11 nmol min⁻¹mg⁻¹ at the
optimal condition of 37°C, pH 4.0 (Figure 5A, Table 2). Furthermore, several divalent
ions (i.e., Mn²⁺, Ca²⁺, Fe²⁺, and Mg²⁺) were added to and removed from the buffer to
check whether AtTLP18.3 is a divalent-ion–dependent phosphatase. The metal-free
AtTLP18.3 showed reduced specific activity, by about 25%. Therefore, the phosphatase
activity of AtTLP18.3 would be maintained by divalent ions but reduced with divalent
ions removed (Figure 4C).

To further determine the phosphatase activity of AtTLP18.3, we examined several
different substrates such as 6,8 difluoro-4-methylumbelliferyl phosphate (DiFMUP) and
pSer and 5 synthetic phosphorylated oligopeptides (Table 2). We used the optimal
condition (37°C, pH 4.0), determined by use of the substrate pNPP. Assay of AtTLP18.3
kinetics showed a classical hyperbolic saturation with pNPP but an allosteric sigmoid
with pSer. The Km and Vmax from the substrate pSer were 47.51 mM and 14.55 nmol
min⁻¹mg⁻¹, respectively (Figure 5B and Table 2). In addition, kinetics assay with the
substrate DiFMUP revealed the classical hyperbolic saturation (Supplemental Figure S2
online).

We tested the phosphatase activity of AtTLP18.3 against 5 synthetic phosphorylated
oligopeptides: Ac-(pT)AILER and Ac-(pT)IALGK are the phosphorylation sites of the
major phosphopeptides of D1 and D2 proteins from Arabidopsis thylakoids (Vener et al.,
RRA(pT)VA and KR(pT)IRR are good substrates of the serine-threonine phosphatases (Donella-Deana et al., 1991); and Ac-RK(pS)AGKPKN is the phosphorylation site of the phosphopeptides from spinach thylakoids (Michel et al., 1991). The highest $K_m$ of phosphopeptide RRA(pT)VA was 11.89 mM. The highest $V_{max}$ of phosphopeptide Ac-RK(pS)AGKPKN was 7.43 nmol min$^{-1}$mg$^{-1}$. The kinetics results for AtTLP18.3 are in Table 2. Thus, AtTLP18.3 functions as an acid phosphatase by removing the phosphate group from pSer or pThr.

**Structure determination of the AtTLP18.3–pSer complex**

The enzymatic activity of AtTLP18.3 could remove the phosphate group from pSer or pThr; therefore, we analyzed the structure of the AtTLP18.3–pSer complex to examine the active site of AtTLP18.3. Crystals of AtTLP18.3 were grown and transferred to the same buffer containing an additional 20 mM pSer for 7 days. After soaking with pSer, the crystal of the AtTLP18.3–pSer complex was diffracted to 2.1 Å resolution (Table 1). The phosphate group of pSer was removed from the structure, and the residue Ser remained in the binding site (Figure 6A). Electron density analysis of the residue Ser showed formation of a hydrogen bond with the residues Val101, Asp102, and Lys112 (Figure 6B). The residues Val101 and Asp102 are conserved, and the residue Lys112 is conserved only in land plants (Figure 2A). We identified a binding site of calcium ion with 6 hydrogen bonds formed by the O atom of Ser85, the carbonyl group and the carboxylic group of Asp103, the carbonyl group of Arg136, and 2 water molecules (Figure 6C).
DISCUSSION

AtTLP18.3 is an auxiliary protein present in the lumen side of the thylakoid membrane and its gene expression follows a circadian rhythm

In the past several years, different proteomic approaches have revealed hundreds of chloroplast proteins, including those with unknown function, located at the thylakoid membrane and lumen (Schubert et al., 2002; Friso et al., 2004; Goulas et al., 2006; Sirpio et al., 2007). These proteomic studies provide a detailed overview of photosynthetic apparatuses and the auxiliary proteins for maintaining the photosystem in the chloroplast. The studies revealed the function of most proteins isolated from the polysome in the thylakoid membrane as involved in photosynthetic apparatuses or translational complexes. Some potential auxiliary proteins with unknown function were also identified. AtTLP18.3 is one of the proteins associated with the polysome, and only a low amount can be found in the thylakoid preparation. Furthermore, the amount of AtTLP18.3 protein remains constant and is not regulated by light (Sirpio et al., 2007).

AtTLP18.3 showed a diurnal rhythm of gene expression under a 16-h light/8-h dark growth photoperiod in Arabidopsis. The transcripts of AtTLP18.3 showed high expression in the daylight and low expression in the dark (Figure 1). In contrast to the gene expression, the protein level remained constant (Sirpio et al., 2007). The reasons for the differential expression might involve posttranscriptional control for the gene expression of AtTLP18.3 and the stability of the protein globular architectures maintaining the amount of AtTLP18.3 protein. Further experiments are needed to confirm this possibility.

AtTLP18.3 contains a novel acid phosphatase domain

Phosphatase activity in chloroplasts has been characterized by previous studies (Sun et al., 1989; Vener et al., 1999; Trotta et al., 2011). The catalytic domain of some kinds of phosphatases, such as homologs of protein phosphatase 2A, may be exposed to the stroma face (Vener et al., 1999). However, the phosphatase located at the thylakoid lumen has not been reported. From our structural comparison with several known
proteins, AtTLP18.3 seemed to be a novel acid phosphatase in the thylakoid lumen. We further analyzed the phosphatase activity of AtTLP18.3 with different substrates, such as pNPP, DiFMUP, pSer, and 5 phosphorylated oligopeptides. AtTLP18.3 showed phosphatase activity with 2 artificial substrates, pNPP and DiFMUP. To explore the possible cellular substrate activity, we confirmed the acid phosphatase activities with pSer and several designed phosphorylated oligopeptides from PSII (Vener et al., 2001). Theoretically, because the phosphorylated oligopeptides are located at the N terminus of PSII in the chloroplast stroma, their dephosphorylation by AtTLP18.3 should not occur in the thylakoid lumen. Further effort is needed to search for substrates in the lumen side for AtTLP18.3. Because AtTLP18.3 exhibited phosphatase activity on pSer and pThr (Table 2), DUF477 should be renamed the thylakoid acid phosphatase (TAP) domain (Figure 2B). As compared with one undefined acid phosphatase with 10.4 μmol/min/mg specific activity of pNPP in thylakoid membranes (Rengasamy et al., 1981), AtTLP18.3 showed much lower phosphatase activity (Table 2), which indicates another kind of acid phosphatase in the thylakoid lumen.

Is AtTLP18.3 involved in the repair cycle of the photosystem?

In the chloroplasts, a transthylakoid proton motive force (pmf) can be established across the thylakoid membrane by photosynthetic proton transfer, and the lumen becomes acidic during photosynthesis (Pottosin and Schonknecht, 1996; Kieselbach et al., 1998). Previous study indicated that the pH of the thylakoid lumen is between 5.8 and 6.5 under normal conditions and decreases to become strongly acidic, < 5, under stress conditions (Kramer et al., 1999). From our results, the gene expression of AtTLP18.3 in the lumen follows a diurnal rhythm, with the protein level constant. In addition, the optimal pH for the acid phosphatase activity of AtTLP18.3 was between 3.5 and 4.5. Thus, the phosphatase activity of AtTLP18.3 in the lumen might be activated in the daylight and under acidic stress. The activity would be deactivated in the dark when the lumen was in a neutral condition. In the stress condition, the more acidic condition might present an activated environment for AtTLP18.3 removing the phosphate group from damaged proteins.
AtTLP18.3 may be involved in regulating the repair cycle of the photosystem (Sirpio et al., 2007). Phosphorylation and dephosphorylation are important regulation mechanisms in a wide range of cellular processes. The thylakoid membrane contains many photosynthetic apparatuses. Many proteins in the PSII reaction center undergo posttranslational phosphorylation under high light radiation. One of the core proteins in the PSII reaction center, D1, is regulated by reversible phosphorylation (Koivuniemi et al., 1995). Phosphorylation of D1 is a signal for migration of the photodamaged PSII core complex from grana membranes to stroma lamellae, and then the photodamaged D1 protein is digested by protease and replaced by new D1 protein. Without dephosphorylation, phosphorylated D1 is a poor substrate for degradation (Ebbert and Godde, 1996). However, the phosphorylation of D1 protein occurs in the N terminus of D1, towards the stroma side of thylakoid membrane. The phosphorylated sites of D1 in the stroma and AtTLP18.3 in the thylakoid lumen will not physically interact because of different localization. This situation raises the question of how the AtTLP18.3 protein can repair the photosystem after photodamage. AtTLP18.3 might not be directly involved in the repair cycle of the photosystem. The thylakoid lumen may contain some unknown phosphoproteins that undergo dephosphorylation by the phosphatase activity of AtTLP18.3.

In conclusion, we used a structure approach to explore the molecular function of AtTLP18.3. The truncated AtTLP18.3 with only the domain of unknown function, DUF477, was crystallized and diffracted at resolution 1.6 Å. Because the native AtTLP18.3 contained no methionine residues, we introduced 2 methionine residues into AtTLP18.3 by site-directed mutagenesis. From the double mutation of AtTLP18.3, the crystal structure of the AtTLP18.3 mutant was resolved by the SAD method. The structure of truncated AtTLP18.3 contains a domain with an $\alpha/\beta$ fold that belongs to a Rossmann fold. Structure comparison indicated that AtTLP18.3 may function as a phosphatase in the thylakoid lumen. We used the standard reagents for phosphatase, such as $p$NPP, DiFMUP, pSer and phosphopeptides of the chloroplast, to confirm the phosphatase activity. Enzymatic kinetics and biochemical analyses revealed an optimal condition for expression at 37°, pH 4, and identified the acid phosphatase activity. Therefore, the DUF477 of AtTLP18.3 is renamed the TAP domain. In addition, the
substrate binding site in the structure of AtTLP18.3 was determined by use of a
AtTLP18.3–pSer complex. The acid phosphatase activity of AtTLP18.3 in the thylakoid
lumen may be associated with dephosphorylation under daylight and stress.
MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh. was used in this study. Seeds of the *CCA1*-*ox* mutant were a gift from Dr. E. M. Tobin (University of California Los Angeles). Seeds were sown in soil, acclimated at 4 °C for 2-4 days and then transferred to a thermo-controlled growth chamber. Seedlings were imbibed and grown under a 16-h light/8-h dark photoperiod at 23 °C and a light intensity of 100-120 μmol m⁻² s⁻¹. For one-week-old seedlings, nutrients were further supplied twice a week.

cDNA Cloning of *Arabidopsis TLP18.3* (*AtTLP18.3*)

The cDNA (*At1g54780*) of *Arabidopsis* (was prepared from total RNA with use of SuperScript™ III (Invitrogen). The DNA fragment of *AtTLP18.3* excluding the N-terminal transit peptide and C-terminal transmembrane domain was amplified from full-length cDNA with the forward TLP-*Bam*HI primer (5’CGACGCGAATTCGCCTCTGAGTTCAATATC 3’) and the reverse TLP-*Xho*I  primer (5’CGATGCCTCGAGTTAACTGAACTGTCCTCGCTT 3’), in which the restriction sites of *EcoR*I and *Xho*I sites were introduced at the 5’ and 3’ ends. The obtained DNA fragment was cloned into a pGEM-T Easy vector (Promega) and amplified in the host of *Escherichia coli* strain DH5. After confirmation by DNA sequencing, the fragment was subcloned into the pGEX6P1 expression vector (GE Healthcare).

Secondary Structure Prediction and Site-directed Mutagenesis

Secondary-structure prediction was performed by use of the PSIPRED protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/) (Jones, 1999; McGuffin et al., 2000) for assigning the topology of *AtTLP18.3*. After prediction, several residues were chosen for methionine substitution based on previous reports (Gassner and Matthews, 1999; Ohmura et al., 2001). Point mutations involved use of the site-directed mutagenesis protocol of whole-plasmid synthesis PCR (Weiner et al., 1994) with the designated
primers (Supplemental Table S1 online) and the plasmid of pGEX6P1-AtTLP18.3 as a template. The above complimentary primers were optimized by use of the web-based primer design program PrimerX (http://bioinformatics.org/primerx/) and synthesized by Mission Biotech Inc. (Taipei, Taiwan). The conditions of PCR for site-directed mutagenesis consisted of a 5-min denaturation at 95°C, followed by 16 cycles of 30 sec at 95°C, 60 sec at 55°C, 5.5 min at 72°C. After amplification, the reactions were digested with DpnI for 1 h at 37°C to remove template plasmid DNA. The mutants containing a single point-mutation were first made individually and expressed as for overexpression of the wild-type protein for checking protein solubility. The mutant with a double-point mutation, residues L128M and I159M, was finally chosen and generated from the soluble proteins. All plasmids containing point mutations were confirmed for substitution of the target residues by DNA sequencing.

**Protein Expression and Purification**

Both the wild-type and mutant with double mutations (L128M and I159M) of AtTLP18.3 were overexpressed in *E. coli* BL21 (DE3). The transformant cells of the wild-type AtTLP18.3 were grown at 37 °C in LB medium containing 100 µg ml⁻¹ ampicillin to optical density (OD₆₀₀ nm) 0.5 to 0.7 before induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. The transformant cells containing the double mutation of AtTLP18.3 were grown in M9 medium. L-Selenomethionine (Se-Met) at 100 µg ml⁻¹ was supplied when A₆₀₀ reached 0.6-0.8, and incubated at 28 °C for 1 h. Finally, the recombinant protein in bacteria cells were induced with 1 mM IPTG at 28 °C for 12 to 16 h. All bacterial cells were harvested and lysed by use of a sonicator (Misonix, Model XL 3000) in the binding buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl). The supernatant containing the recombinant protein was then collected by centrifugation at 12000×g for 30 min at 4 °C. The glutathione-S-transferase (GST) fusion protein was purified by use of a GSTrap FF column with an Äkta Prime fast protein liquid chromatography (FPLC) system (GE Healthcare). After washing the GST affinity column with 10 column volumes of the binding buffer, the recombinant protein was further eluted with elution buffer (50 mM Tris-HCl pH 8.8, 10 mM reduced glutathione). The eluted protein solutions were dialysed with the cleavage buffer (50 mM Tris-HCl pH 7.0, 150
mM NaCl, 1 mM DTT) for equilibration and then cleaved by use of Precission protease (GE Healthcare) with 10 U/mg fusion protein at 4 °C for 16 h. After cleavage, the Precission protease and GST protein were removed by passage through a GSTrap FF column. The purified recombinant AtTLP18.3 protein was concentrated by ultrafiltration and dialysed with the dialysis tube (Amicon Ultra-4; Millipore) and a buffer containing 20 mM Tris-HCl pH 7.0, 150 mM NaCl. The expression and purification of AtTLP18.3 by 12.5% SDS-PAGE are shown in Supplemental Figure S1 online. The Se-Met protein was purified with the same protocol as for the wild-type protein, except for the addition of 1 mM Tris (2-carboxyethyl) phosphine (TCEP).

**Northern Blot Analysis**

Total RNA was extracted with the REzol™ C&T reagent and analyzed on a 1.2% formaldehyde agarose gel. After electrophoresis, the RNA was transferred from the agarose gel to a Nylon membrane, and then the membrane was hybridized with a digoxigenin-11-dUTP–labeled probe. Finally, the signal was captured by use of a LAX-3000 Image System (Fuji). For the CCA1 1 northern blot probe, CCA1-F (5’GAGCAAGGACCTAGACTTATCCG 3’) and CCA1-R (5’GGAAGGCAATTCGGACCTCG 3’) were designed for the digoxigenin-11-dUTP–labeled probe. The primers TLP-BamHI and TLP-XhoI were used for the northern blot probe of AtTLP18.3.

**Crystallization, Data Collection, and Structure Determination**

The wild-type and Se-Met–labeled AtTLP18.3 proteins were crystallized by the hanging-drop vapor diffusion method (McPherson, 1990) at 23°C. Most crystals were grown in 0.2 M sodium acetate trihydrate, 0.1 M sodium cacodylate (pH 6.5) and 25-30 % (w/v) polyethylene glycol 4000 within 2 weeks. Crystals of Se-Met–containing protein were grown under similar conditions with slightly lower pH 6.0 and the addition of 2 mM TCEP to all solutions. For the AtTLP18.3–pSer complex, a soaking method was used. The crystals of wild-type AtTLP18.3 were first grown as described above and then transferred to the above buffer containing 20 mM pSer for 7 days.
Crystals were rapidly swept through mother liquor containing 25% (v/v) glycerol as a cryoprotectant and flash-cooled in liquid nitrogen at 100 K. The diffraction data were collected by synchrotron radiation on the SPXF beamlines BL13B1 and BL13C1 equipped with CCD detectors (Q315 and Q210, ADSC) at the National Synchrotron Radiation Research Center (NSRRC, Taiwan). All diffraction intensities were integrated and scaled by use of the HKL2000 software package (Otwinowski and Minor, 1997). The crystal structure of AtTLP18.3 was determined by single-wavelength anomalous dispersion (SAD), and 2 Se sites were located with use of the SOLVE software (Terwilliger and Berendzen, 1999). The preliminary coordination was determined by the RESOLVE program. Manual model rebuilding involved use of the program Coot (Emsley and Cowtan, 2004), alternating with the structure refinement with the software suite Crystallography & NMR System (CNS) (Brunger et al., 1998) with 5% of the observed reflections randomly selected and the R\text{free} value calculated. The crystallography statistics are in Table 1. All ribbon diagrams were prepared by use of PyMOL (DeLano, 2004; DeLano and Lam, 2005).

Phosphatase Activity Assay

The activity of AtTLP18.3 to hydrolyze organophosphate substrates was analyzed with use of the substrates \( \text{pNPP} \) (New England Biolabs), DiFMUP (Molecular Probes), pSer (Sigma-Aldrich), and 5 synthetic phosphorylated oligopeptides (Kelowna International Scientific, Taipei) with following composition, where (pX) indicates the phosphorylated residue and Ac- indicates the N-terminal acetylation of the peptides: Ac-(pT)AILER, Ac-(pT)IALGK, RRA(pT)VA, KR(pT)IRR, and Ac- RK(pS)AGKPKN.

Enzymatic kinetics of AtTLP18.3 were calculated with the substrates at a series of concentrations at 37°C. The incubated solution contained 15 \( \mu \)g purified AtTLP18.3, 50 mM sodium acetate buffer (pH 4.0), and the substrates to a total volume of 0.1 ml. All reactions were initiated by adding purified AtTLP18.3 and incubating for 60 min at 37°C, with a blank reaction with AtTLP18.3 omitted. For the substrate \( \text{pNPP} \), the reactions were terminated by the addition of 0.25 ml of 500 mM NaOH, and absorbance was measured at 405 nm by use of the spectrophotometer GeneQuant 1300 (GE Healthcare).
For the substrate DiFMUP, the fluorescence of the product DiFMU was detected at 358 nm excitation and 455 nm emission. The fluorescence was recorded by use of the spectrophotometer Beckman DU640B. For the substrates pSer and phosphorylated oligopeptides, inorganic phosphate was measured colorimetrically by the modified malachite green method (Fisher and Higgins, 1994). The malachite green-ammonium molybdate reagent containing 0.045% (w/v) malachite green in 6 N H<sub>2</sub>SO<sub>4</sub>, 7.5% (w/v) ammonium molybdate in 6 N H<sub>2</sub>SO<sub>4</sub>, and 11% Tween 20 in the ratio 1:0.68:0.02 added to the reaction buffer. Absorbance was measured at 650 nm to determine levels of inorganic phosphate generation as compared with the standard curve of KH<sub>2</sub>PO<sub>4</sub>. Finally, the optimal activity conditions of AtTLP18.3 were determined according to the results of pNPP used as a substrate. The optimal pH for pNPP hydrolysis was measured with 50-mM concentrations of the buffers with sodium citrate (pH 2 to 3), sodium acetate (pH 3.5 to 5), MES-NaOH (pH 5.5 to 6), and Tris-HCl (pH 7.0). The effect of temperature was determined in the range of 10 to 55 °C.

Accession Numbers for Gene, Protein Sequences and Protein Structures

The gene sequence of AtTLP18.3 is in the Arabidopsis Information Resource database (TAIR; accession no. AT1G54780). The accession numbers of amino acid sequences in the National Center for Biotechnology Information (NCBI) are NP_564667 for AtTLP18.3, NP_001055486 for *Oryza sativa* (japonica cultivar-group), ABF21087 for *Selaginella tamariscina*, XP_001420826 for *Ostreococcus lucimarinus* CCE9901, NP_441552 for *Synechocystis* sp. PCC 6803, and YP_002378642 for *Cyanothece* sp. PCC 7424. Amino acid sequences were aligned by use of ClustalW (http://www.ch.embnet.org/software/ClustalW.html). Figure 2 was prepared by use of the web-based ESPript 2.2 (http://espript.ibcp.fr/ESPript/ESPript/). All structural factors and coordinates have been deposited in the Protein Data Bank (PDB) with PDB code 3PTJ for the SeMet AtTLP18.3, 3PVH for the wild-type AtTLP18.3 and 3PW9 for the AtTLP18.3–pSer complex.

SUPPLEMENTAL MATERIALS

The following materials are available in the online version of this article.
Supplemental Table S1. Primers for site-directed mutagenesis.

Supplemental Figure S1. Expression and purification of AtTLP18.3 Protein.

Supplemental Figure S2. Enzymatic kinetics for phosphatase activity assay with the substrate DiFMUP.
ACKNOWLEDGEMENTS

The financial support of this work was from National Science Council, Taiwan to Y. S. Cheng (NSC. 98-2313-B-002-059-MY2). Portions of this research were carried out at the National Synchrotron Radiation Research Center, a national user facility supported by the National Science Council of Taiwan, ROC. The Synchrotron Radiation Protein Crystallography Facility is supported by the National Research Program for Genomic Medicine.
FIGURE LEGENDS

Figure 1. Cis-acting regulatory and expression pattern of AtTLP18.3 gene.
(A) Cis-acting regulatory element analyzed by PLACE and PlantCare. Nucleic acid sequence analysis revealed the sugar-repressive element (S), the CCA1 binding site (C), the phytochrome-regulation element (P), and the GA-responsive element (G) within 1500 bp upstream of the translation start codon of the AtTLP18.3 gene. The block indicates a TATA box. The bar represents 100 bp of nucleic acids.
(B) Expression pattern of AtTLP18.3 in wild-type Arabidopsis.
(C) Comparison of the expression pattern of AtTLP18.3 in wild-type plants and the CCA1-ox mutant. The bars represent standard errors.
(D) Expression pattern of AtTLP18.3 in CCA1-ox mutant. Expression pattern of wild-type (B) and CCA1-ox mutants (D); the duration under light, L, and dark, D, are denoted at the top of the first panel. Open bars denote lighted intervals and solid bars darkness. The expression of CCA1 is an internal control. The expression pattern of AtTLP18.3 is shown. Ethidium bromide (EtBr) is a loading control. The signal of AtTLP18.3 on northern blots was normalized with 28S ribosomal RNA.

Figure 2. Sequence alignment of AtTLP18.3 and its homologs.
(A) Structure-based multiple sequence alignment of AtTLP18.3 among plants, algae and cyanobacteria homologues. The homologs of AtTLP18.3 show conserved residues and similar domain architecture. Positions of identically conserved residues are shown in white on red, and regions of similarly conserved residues are boxed. The secondary structure elements are depicted above the sequence alignment: α helices are denoted by coils and β strands by arrows. Representation of secondary structure elements and numbering is based on the AtTLP18.3 structure. From sequence alignment, the predicted transit peptide and transmembrane region are delimited in the corresponding boxes. Two point mutations, L128M and I159M, were introduced for the single-wavelength anomalous dispersion method by site-directed mutagenesis. The residues for Ser binding are shown by a green triangle and calcium ion binding a yellow circle. Species for the sequences are A. thaliana, Arabidopsis thaliana; O. sativa, Oryza sativa (japonica
cultivar-group); *S. tamariscina, Selaginella tamariscina; Ostreococcus, Ostreococcus
lucimarinus* CCE9901; *Synechocystis, Synechocystis* sp. PCC 6803; and *Cyanothece, Cyanothece*
sp. PCC 7424.

(B) The mature form of AtTLP18.3 in the thylakoid lumen. From sequence analysis, the
mature AtTLP18.3 could be predicted with 2 major regions: a domain of unknown
function (DUF477) and a transmembrane α-helix (TMH).

**Figure 3.** Overall structure and topology diagram of AtTLP18.3.

(A) The crystal structure of AtTLP18.3 was built from residues 83 to 235, which is the
thylakoid acid phosphatase (TAP) domain only. The structure contains the mixed
α/β Rossmann fold with a parallel four-stranded twisted β-sheet (topology β1, β2, β3, β4)
flanked by 3 helices on one side (α1, α3) and 3 helices on the other side (α2, α6, α7).

(B) The solution structure of Mannitol-specific cryptic phosphotransferase enzyme II A
CmtB (PDB ID: 2OQ3) from *Escherichia coli* shares a similar structure and topology
with AtTLP18.3. In comparison to CmtB, the RMSD of AtTLP18.3 is 3.48 Å, with a
lower amino acid sequence identity of 12%. AtTLP18.3 also shows a similar structure,
with a low structural similarity to other phosphatases. Secondary structure elements are
distinguished by color: α helix by red, β sheet by green and loops by yellow, respectively.

(C) Electrostatic surface potential contoured from -10 kT (red) to 10 kT (blue). Two
views related by a 180° rotation.

**Figure 4.** Biochemical characterization of AtTLP18.3 phosphatase activity.

(A) Characterization of AtTLP18.3 for optimal pH. Optimal pH was between 3.5 and 4.5.

(B) Characterization for optimal temperature. Maximal activity was found at 37°C.
Phosphatase activity with *pNPP* used as a substrate. Phosphatase activity was assayed by
incubating samples of the protein and 7.5 mM *pNPP* in 0.1 M sodium acetate buffer (pH
4.0). Reactions were initiated by adding purified AtTLP18.3 and incubated for 30 min at
room temperature.

(C) Effect of divalent metal ion on the phosphatase activity. Divalent ions were removed
by adding EDTA to all buffers during purification. The metal-free AtTLP18.3 reduced
the specific activity of AtTLP18.3 by about 25%. However, similar activity levels were observed in the presence or absence of divalent ions. Bars represent the standard errors.

**Figure 5.** Enzymatic kinetics of phosphatase activity with 2 different substrates, pNPP and pSer.

(A) Michaelis-Menten plot of AtTLP18.3 with pNPP used as the substrate. The $K_m$ for pNPP is 42.56 mM, and the $V_{max}$ is 8.11 nmol min$^{-1}$mg$^{-1}$.

(B) Michaelis-Menten plot of AtTLP18.3 with pSer (O-phospho-L-serine) used as the substrate. The $K_m$ for pSer is 47.51 mM, and the $V_{max}$ is 14.55 nmol min$^{-1}$mg$^{-1}$. The kinetics assay of AtTLP18.3 shows a classical hyperbolic saturation with pNPP but an allosteric sigmoid with pSer. Bars represent the standard errors.

**Figure 6.** The structure of the AtTLP18.3–pSer complex.

(A) The binding site of AtTLP18.3 can be identified with a dephosphorylated pSer (O-phospho-L-Serine) and a calcium ion in the structure of AtTLP18.3. A bound calcium ion is represented by a yellow sphere, and a serine is represented by a stick.

(B) The substrate binding site of AtTLP18.3. The residue Ser is contoured by a 1.0 $\sigma$ 2Fo-Fc map and is surrounded by the residues V101, D102, and K112. The yellow dashes represent hydrogen bonds.

(C) Hydrogen bonding for calcium ion in the AtTLP18.3. Six coordinates for calcium ion (Ca) were formed by an O atom of S85, a carbonyl group and a carboxylic group of D103, a carbonyl group of R136, and 2 water molecules (denoted by wa). The yellow dashes represent hydrogen bonds.
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Table 1. Crystallography statistics and refinement for AtTLP18.3

|                                        | TLP         | TLPM45     | TLP–pSer complex |
|----------------------------------------|-------------|------------|-----------------|
| Beamlines at NSRRC                     | 13B1        | 13B1       | 13C1            |
| **Diffraction data statistics**        |             |            |                 |
| Space group                            | $P_{2_1}2_1$ | $P_{2_1}2_1$ | $P_{2_1}2_1$    |
| Cell constants (Å and deg.)            | $a=46.98$, $b=49.84$, $c=76.67$; $\alpha=\beta=\gamma=90^\circ$ |
| Wavelength (Å)                         | 0.97315     | 0.9788     | 0.9762          |
| Resolution range (Å)                   | 30.0-1.6    | 30.0-2.6   | 30.0-2.1        |
| Observed reflections                   | 343,890     | 57,179     | 58,760          |
| Unique reflections                     | 24,243      | 5,797      | 10,968          |
| Completeness (%)                       | 99.3 (100.0)| 97.8 (99.6)| 99.5 (99.9)     |
| $I/\sigma(I)$                          | 58.6 (8.7)  | 30.8 (38.4)| 26.4 (3.7)     |
| $R_{merge}$ (%)                        | 3.5 (31.6)  | 4.6 (6.2)  | 6.3 (49.6)      |
| **Phasing statistics**                 |             |            |                 |
| Number of Se sites                     | 2           |            |                 |
| Signal/Noise                           | 1.55        |            |                 |
| Figure of merit                        | 0.42        |            |                 |
| **Refinement statistics**              |             |            |                 |
| Resolution range (Å)                   | 30.0-1.6    | 30.0-2.6   | 30.0-2.1        |
| Reflections (working/test)            | 21,263/2,346| 9,945/552  | 9,319/1,068     |
| $R_{work}/R_{free}$ (%)               | 19.5/23.0   | 19.9/27.0  | 18.9/24.8       |
| Number of atoms                        |             |            |                 |
| Protein atoms                          | 1,174       | 1,174      | 1,173           |
| Solvent atoms                          | 180         | 67         | 150             |
| RMS deviations                         |             |            |                 |
| Bond length (Å)                        | 0.022       | 0.015      | 0.012           |
| Bond angle (degree)                    | 2.2         | 1.6        | 1.5             |
| Average B-factor (Å²)                  |             |            |                 |
| B-factor (protein)                     | 28.9        | 31.3       | 39.7            |
| B-factor (solvent)                     | 51.5        | 37.1       | 57.8            |
| B-factor (ligand)                      |             |            |                 |
| Ramachandran plot (%)                  |             |            |                 |
| Most favored                           | 91.7        | 91.7       | 93.2            |
| Additional allowed                     | 7.5         | 8.3        | 6.8             |
| Generously allowed                     | 0.8         | 0.0        | 0.0             |
| Disallowed                             | 0.0         | 0.0        | 0.0             |

*R_{merge} = \frac{\Sigma_h \Sigma_i | F_{h,i} - \langle F_i \rangle |}{\Sigma_h \Sigma_i F_{h,i}} $ where $\langle F_i \rangle$ is the mean intensity of $i$ observations for a given reflection $h$. 

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Table 2. Kinetics of phosphatase activity of AtTLP18.3

| Substrates         | $K_m$    | $V_{max}$# |
|--------------------|----------|------------|
| pNPP               | 42.56 mM | 8.10       |
| pSer               | 47.51 mM | 14.55      |
| DiFMUP             | 49.56 μM | -          |
| Phosphopeptides    |          |            |
| Ac-(pT)AILER       | 12.49 mM | 3.15       |
| Ac-(pT)IALGK       | 16.57 mM | 4.73       |
| RRA(pT)VA          | 11.89 mM | 4.97       |
| KR(pT)IRR          | 12.56 mM | 4.02       |
| Ac-RK(pS)AGKPKN    | 13.88 mM | 7.43       |

$#\quad$ The unit of $V_{max}$ is nmol min$^{-1}$ mg$^{-1}$
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Expression pattern of wild-type (B) and CCA1-ox mutants (D); the duration under light, L, and dark, D, are denoted at the top of the first panel. Open bars denote lighted intervals and solid bars darkness. The expression of CCA1 is an internal control. The expression pattern of AtTLP18.3 is shown. Ethidium bromide (EtBr) is a loading control. The level of AtTLP18.3 on northern blots was normalized with 28S ribosomal RNA.
Figure 2. Sequence alignment of AtTLP18.3 and its homologs.

(A) Structure-based multiple sequence alignment of AtTLP18.3 among plants, algae, and cyanobacteria homologues. The homologs of AtTLP18.3 show conserved residues and similar domain architecture. Positions of identically conserved residues are shown in white on red, and regions of similarly conserved residues are boxed. The secondary structure elements are depicted above the sequence alignment: α helices are denoted by coils and β strands by arrows. Representation of secondary structure elements and numbering is based on the AtTLP18.3 structure. From sequence alignment, the predicted transit peptide and transmembrane region are delimited in the corresponding boxes. Two point mutations, L128M and I159M, were introduced for the single-wavelength anomalous dispersion method by site-directed mutagenesis. The residues for Ser binding are shown by a green triangle and calcium ion binding a yellow circle. Species for the sequences are A. thaliana, Arabidopsis thaliana; O. sativa, Oryza sativa (japonica cultivar-group); S. tamariscina, Selaginella tamariscina; Ostreococcus, Ostreococcus lucimarinus CCE9901; Synechocystis, Synechocystis sp. PCC 6803; and Cyanothecae (cyanoecys sp. PCC 7792).

(B) The mature form of AtTLP18.3 in the thylakoid lumen. From mature form, the TAP domain, which is involved in thylakoid targeting, and DUF477 domain are denoted. A truncated form is also shown.
Figure 3. Overall structure and topology diagram of AtTLPI8.3.
(A) The crystal structure of AtTLPI8.3 was built from residues 83 to 235, which is the thylakoid acid phosphatase (TAP) domain only. The structure contains the mixed α/β Rossmann fold with a parallel four-stranded twisted β-sheet (topology β1, β2, β3, β4) flanked by 3 helices on one side (α1, α3) and 3 helices on the other side (α2, α6, α7).
(B) The solution structure of Mannitol-specific cryptic phosphotransferase enzyme II A CmtB (PDB ID: 2Q3) from Escherichia coli shares a similar structure and topology with AtTLPI8.3. In comparison to CmtB, the RMSD of AtTLPI8.3 is 3.48 Å, with a lower amino acid sequence identity of 12%. AtTLPI8.3 also shows a similar structure, with a low structural similarity to other phosphatases. Secondary structure elements are distinguished by color: α-helix in red, β-sheet in green and loops in yellow, respectively.
(C) Electrostatic surface potential contoured from -10 kT (red) to 10 kT (blue). Two views related by a 180° rotation.
Figure 4. Biochemical characterization of AtTLP18.3 phosphatase activity.

(A) Characterization of AtTLP18.3 for optimal pH. Optimal pH was between 3.5 and 4.5.

(B) Characterization for optimal temperature. Maximal activity was found at 37°C. Phosphatase activity with pNPP used as a substrate. Phosphatase activity was assayed by incubating samples of the protein and 7.5 mM pNPP in 0.1 M sodium acetate buffer (pH 4.0). Reactions were initiated by adding purified AtTLP18.3 and incubated for 30 min at room temperature.

(C) Effect of divalent metal ion on the phosphatase activity. Divalent ions were removed by adding EDTA to all buffers during purification. The metal-free AtTLP18.3 showed 25% lower specific activity of AtTLP18.3 by about 25%. However, similar activity levels were observed in the presence or absence of divalent ions. Bars represent the standard errors.
Figure 5. Enzymatic kinetics of phosphatase activity with 2 different substrates, pNPP and pSer.

(A) Michaelis-Menten plot of AtTLP18.3 with pNPP used as the substrate. The Km for pNPP is 42.56 mM, and the Vmax is 8.11 nmol min⁻¹mg⁻¹.

(B) Michaelis-Menten plot of AtTLP18.3 with pSer (O-phospho-L-serine) used as the substrate. The Km for pSer is 47.51 mM, and the Vmax is 14.55 nmol min⁻¹mg⁻¹. The kinetics assay of AtTLP18.3 shows a classical hyperbolic saturation with pNPP but an allosteric sigmoid with pSer. Bars represent the standard errors.
Figure 6. The structure of the AtTLP18.3-pSer complex.

(A) The binding site of AtTLP18.3 can be identified with a dephosphorylated pSer (O-phospho-L-Serine) and a calcium ion in the structure of AtTLP18.3. A bound calcium ion is represented by a yellow sphere, and a serine is represented by a stick.

(B) The substrate binding site of AtTLP18.3. The residue Ser is contoured by a 2.0 σ 2Fo-Fc map and is surrounded by the residues V101, D102, and K112. The yellow dashes represent hydrogen bonds.

(C) Hydrogen bonding for calcium ion in the AtTLP18.3. Six coordinates for calcium ion (Ca) were formed by an O atom of S85, a carbonyl group and a carboxylic group of D103, a carbonyl group of R136, and 2 water molecules (denoted by wa). The yellow dashes represent hydrogen bonds.