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SHORT COMMUNICATION

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AtPAP2 modulates the import of the small subunit of Rubisco into chloroplasts

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ABSTRACT

Arabidopsis thaliana purple acid phosphatase 2 (AtPAP2) is the only phosphatase that is dual-targeted to both chloroplasts and mitochondria. Like Toc33/34 of the TOC and Tom 20 of the TOM, AtPAP2 is anchored to the outer membranes of chloroplasts and mitochondria via a hydrophobic C-terminal motif. AtPAP2 on the mitochondria was previously shown to recognize the presequences of several nuclear-encoded mitochondrial proteins and modulate the import of pMORF3 into the mitochondria. Here we show that AtPAP2 binds to the small subunit of Rubisco (pSSU) and that chloroplast import experiments demonstrated that pSSU was imported less efficiently into *pap2* chloroplasts than into wild-type chloroplasts. We propose that AtPAP2 is an outer membrane-bound phosphatase receptor that facilitates the import of selected proteins into chloroplasts.

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AtPAP2; chloroplast; outer membrane; protein import; purple acid phosphatase; Rubisco; transit peptide

Many nuclear-encoded precursor proteins are translocated into chloroplasts and mitochondria to perform their functions. Translocons at the outer and inner membranes of the chloroplasts (TOC/TIC) and mitochondria (TOM/TIM) form delicate import machineries for the import of precursor proteins into chloroplasts and mitochondria.[1] Multiple layers of control, including reversible phosphorylation at the transit peptides/presequenes of preproteins, are involved in fine tuning the translocation processes.[2,3] Cytosolic STY kinases were shown to phosphorylate the Ser34 residue of the transit peptide of tobacco small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, SSU)[4,5] and the Thr20/Ser27/Thr35 residues of the presequence of Multiple Organellar RNA Editing Factor 3 (pMORF3),[3] which are then bound by 14-3-3.[3,6] NaF, a broad-range phosphatase inhibitor, inhibits the importing of pSSU and pMORF3 into chloroplasts and mitochondria, respectively. This suggests that the dephosphorylation of pSSU/pMORF3 at their transit peptide/presequence is required before translocation.[2,7]

A number of receptors on the outer membranes of chloroplasts and mitochondria are responsible for binding to precursor proteins in the cytosol, including Toc33/34, Toc64, and Toc159 of TOC in chloroplasts;[8] and Tom20 and OM64 of TOM in mitochondria.[9,10] While both Toc34 and Toc64 recognize the pSSU transit peptide but not mature SSU (mSSU),[8] these proteins prefer different forms of pSSU. Free, non-phosphorylated pSSU binds to the non-tetratricopeptide repeat motif of Toc64. However, because 14-3-3 competes with Toc64 for pSSU binding, phosphorylated pSSU, complexed with 14-3-3, prefers Toc34 to Toc64.[8] A model for pSSU recognition and TOC translocation involving Toc34 and Toc159 has been proposed.[8] The pSSU transit peptide, which is phosphorylated at Ser34 and complexed with 14-3-3, can bind to Toc34. The N-terminal part of the transit peptide then binds to Toc159 to form a trimeric complex. The hydrolysis of GTP on Toc34 dissociates it from the complex so that some unknown phosphatase(s) can dephosphorylate P-Ser34 on pSSU. The hydrolysis of GTP on Toc159 then pushes the preprotein through the translocation channel, Toc75.

Similar to a number of protein receptors of the TOC and TOM complexes, *Arabidopsis thaliana* purple acid phosphatase 2 (AtPAP2) is anchored to the outer membranes of plastids and mitochondria through a hydrophobic transmembrane C-terminal motif (TMD/CT).[11,12] AtPAP2 was shown to interact with the presequences of a number of pMORF proteins, and the import of STY8-treated, presequence-phosphorylated pMORF3 into mitochondria isolated from *AtPAP2* T-DNA line was slower than in mitochondria isolated from the wild-type plants.[3] The goal of this study was to examine if AtPAP2 also interacts with pSSU and modulates its import into chloroplasts.

AtPAP2 on the outer membrane of chloroplasts recognizes pSSU

AtPAP2 was shown to interact with the full-length sequence of pMORF3 but not with the mature protein of MORF (mMORF3), indicating that it recognizes the presequence of pMORF3.[3] To examine whether AtPAP2 interacts with AtSSU1B (AT5G38430), yeast 2-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays were carried out. For Y2H, the coding sequence of the mature bait protein AtPAP2 (25-613a.a.), lacking its N-terminal peptide and C-terminal transmembrane motif (P2NC), was fused to the

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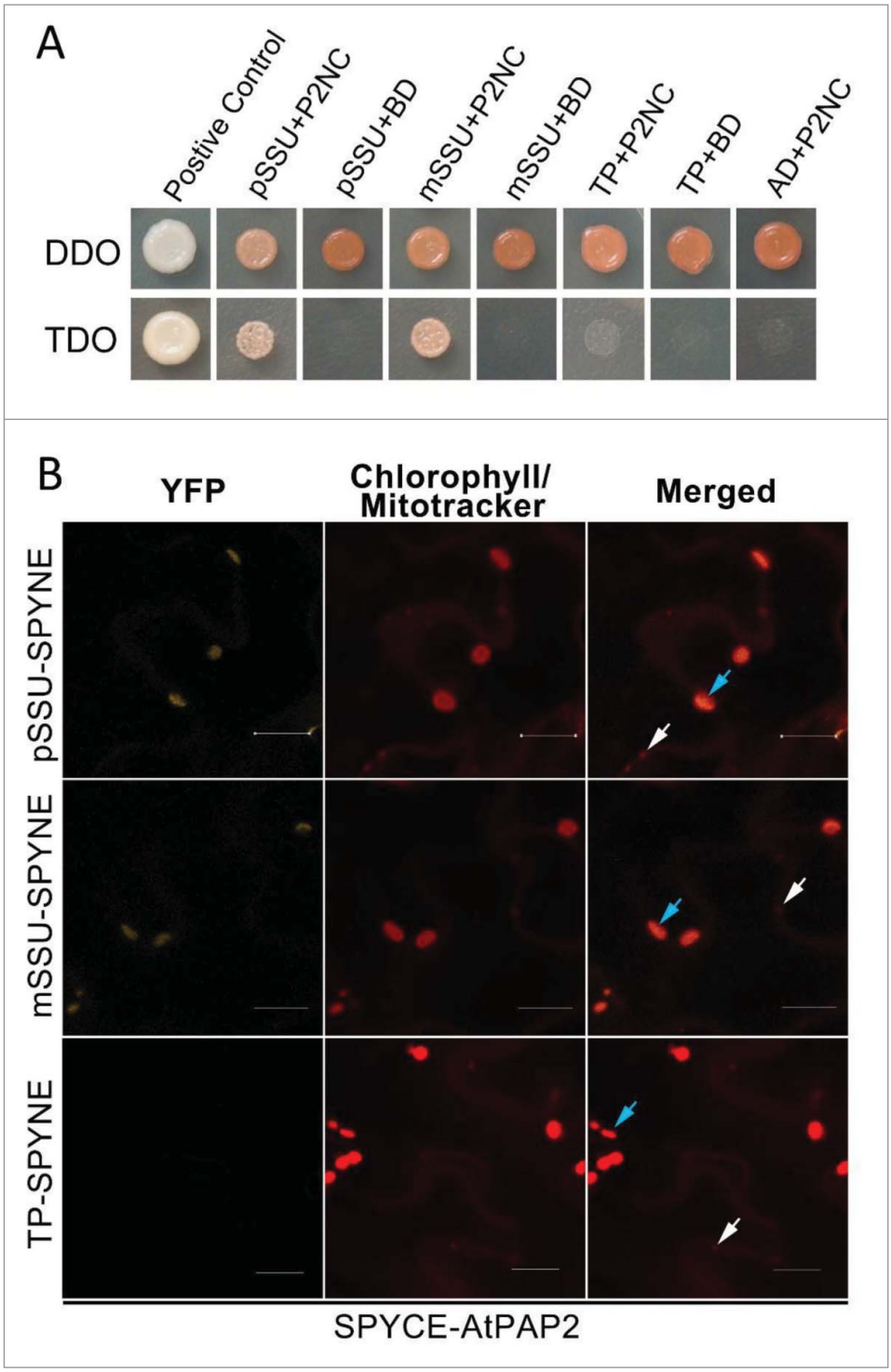

Figure 1. AtPAP2 on outer chloroplast membrane binds pSSU. (A) In Y2H assays, mature AtPAP2 protein without the signal peptide and C-terminus (a.a. 25–613, P2NC) interacted with the full-length SSU (pSSU, a.a. 1–181) and the mature SSU (mSSU, a.a 42–181) but not the transit peptide of SSU (TP, a.a. 1–50 of pSSU). (B) In BiFC assays, full-length AtPAP2 interacted with pSSU and mSSU in chloroplasts. Mitochondria were marked using MitoTracker® Red CMXRos (Life Technology, USA). The locations of the chloroplasts and mitochondria are labeled with blue and white arrows, respectively.

C-terminus of the GAL4 DNA-binding domain (BD) domain of the pGBKT7 vector (Clontech Laboratories, Japan). Full-length AtSSU1B (pSSU, a.a. 1–181), mature SSU (mSSU, a.a 42–181), and the transit peptide of SSU (TP, a.a. 1–50 of pSSU) were fused to the DNA-activating domain (AD) in the pGADT7 plasmids. The primers that were used in this study are listed in Table 1. Y2H Gold yeast cells were co-transformed with both bait and prey constructs using the lithium acetate method and plated onto double dropout (DDO, -Leu/-Trp) and triple dropout (TDO, -Leu/-Trp/-His) agar plates (Clontech Laboratories, Japan). As shown in Fig. 1A, AtPAP2 was found to interact with the pSSU and mSSU but not the transit peptide of pSSU. To confirm the results in plant systems, BiFC was carried out.

Table 1. Primer list.

Vector (Purpose)	Construct (AGI code)	[1]Primer Sequence (5′-3′)
pGBKT7 (Y2H)	P2NC (25-613aaAtPAP2) (AT1G13900)	F: TTCTCATATGACCATTTCAATTTCCCC R: GCATGTCGACCAGCATTAGATTCTGATTTTC
pGADT7 (Y2H)	pSSU (1-181aaSSU1B) (AT5G38430)	F: ATTACATATGGCTTCCTCTATGCTCTCCT R: ATTACTCGAGAGCATCAGTGAAGCTTGGGG
pGADT7 (Y2H)	mSSU (42-181aaSSU1B) (AT5G38430)	F: ATTCCATATGGACATTACTTCCATCACAAGC R: ATTACTCGAGAGCATCAGTGAAGCTTGGGG
pGADT7 (Y2H)	TP (1-50aaSSU1B) (AT5G38430)	F: ATTACATATGGCTTCCTCTATGCTCTCCT R: ATTACTCGAGCCCATTGCTTGTGATGGAAG
SPYCE (BiFC)	AtPAP2 (1-656aa) (AT1G13900)	F: ATCGACTAGTATGATCGTTAATTTCTCTTTCTTC R: GAATACTAGTTTATGTCTCCTCGTTCTTGACTG
SPYCE (BiFC)	Multiple cloning site [2]MCS	F: ATTAGAGCTCGTTAACCGGGCTCAGGCCT R: ATTAGAGCTCCCCGGGAGCGGTACCCTC
SPYCE (BiFC)	[2]YFP^C	F: TAACTCTAGAATGTACCCATACGATGTTCCAG R: ATTAGAGCTCCTTGTACAGCTCGTCCATG
SPYNE (BiFC)	pSSU (1-181aaSSU1B) (AT5G38430)	F: ATTATCTAGAATGGCTTCCTCTATGCTCTCCT R: ATTACTCGAGAGCATCAGTGAAGCTTGGGG
SPYNE (BiFC)	mSSU (42-181aaSSU1B) (AT5G38430)	F: ATTCTCTAGAATGGACATTACTTCCATCACAAGC R: ATTACTCGAGAGCATCAGTGAAGCTTGGGG
SPYNE (BiFC)	TP (1-50aaSSU1B) (AT5G38430)	F: ATTATCTAGAATGGCTTCCTCTATGCTCTCCT R: ATTACTCGAGCCCATTGCTTGTGATGGAAG
pET28a	STY8 (AT2G17700)	F: CTTACCATGGCGATGACGATCAAAGATGAGTCGGAGAGTT R: CATAGAGCTCAGCACTACGACGTTTACCTCTTTCATTATCTG
pET28a	pSSU (1-181aaSSU1B) (AT5G38430)	F: CTTATCATGACGATGGCTTCCTCTATGCTCTCC R: CATAGAGCTCGAAGCATCAGTGAAGCTTGGG
pET28a	mSSU (42-181aaSSU1B) (AT5G38430)	F: CATAGAGCTCGAAGCATCAGTGAAGCTTGGG R: CATAGAGCTCGAAGCATCAGTGAAGCTTGGG
pET28a	pSSUm (1-181aaS32A)	Overlapping F: GTTTGAAGTCATCCGCTGCTTTCCCGGTC Overlapping R: GACCGGGAAAGCAGCGGATGACTTCAAAC

[1] Restricted sites and mutation sites are indicated by underline.
[2]Primers for MCS and YFP^C were used to create a SPYCE vector that can fuse YFP^C to the N-terminus of AtPAP2 so that the native C-terminal hydrophobic motif of AtPAP2 can anchor AtPAP2 to the outer membranes of chloroplasts and mitochondria.

The full-length coding sequence of the bait protein AtPAP2 was fused to the C-terminus of YFPC on the SPYCE vector, while the coding sequences of pSSU, mSSU, or its transit peptide were fused to the N-terminus of the SPYNE containing YFPN 14. Both the prey and bait plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, and the bacteria were infiltrated into the epidermal cell layers of tobacco leaves as previously described.[13] After

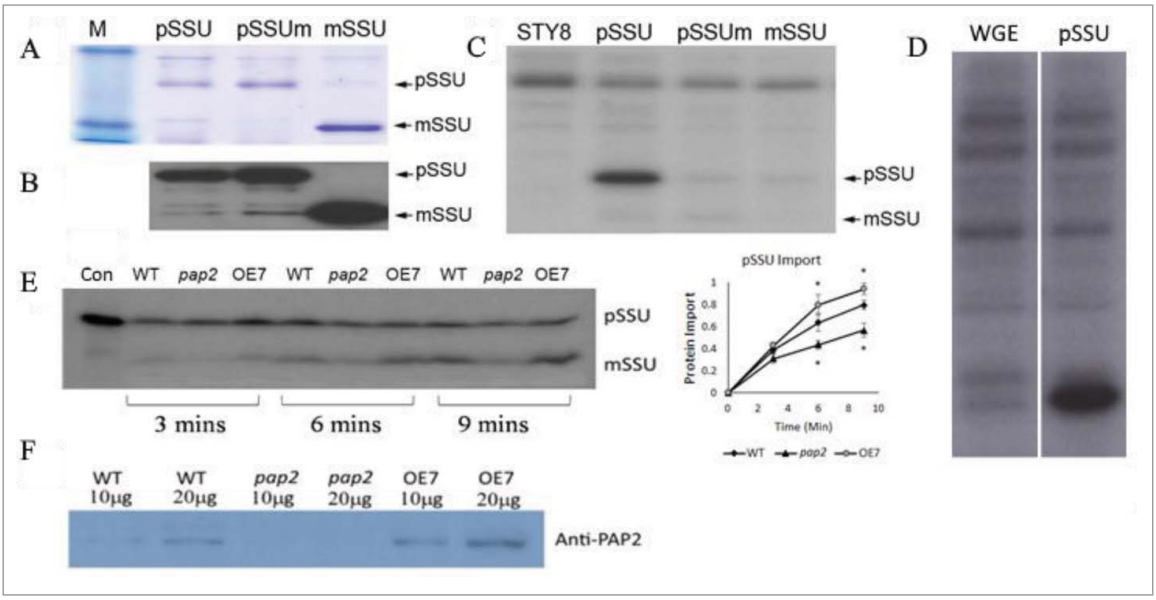

Figure 2. Import of [^{35}S] labeled pSSU into chloroplasts. (A) Recombinant AtSSU1B (pSSU), AtSSU1B-S32A (pSSUm), and mSSU were purified by His-tag chromatography. (B) The identities of pSSU, pSSUm, and mSSU were confirmed using anti-pSSU antibodies (Agrisera). (C) Only pSSU, but not mSSU or pSSUm, was phosphorylated by recombinant STY8 kinase. (D) Recombinant pSSU can be phosphorylated by WGE. (E) Import of WGE-synthesized AtSSU1B into chloroplasts. Con is the radiolabeled AtSSU1B control. The experiment was repeated 3 times with similar results. The import rate of pSSU into OE7 chloroplasts is significantly higher than that of the WT ($^*P \leq 0.05$) while the import rate into *pap2* chloroplasts is significantly lower. (F) Western blot of the proteins isolated from the chloroplasts of WT, *pap2*, and OE7 plants using anti-AtPAP2.

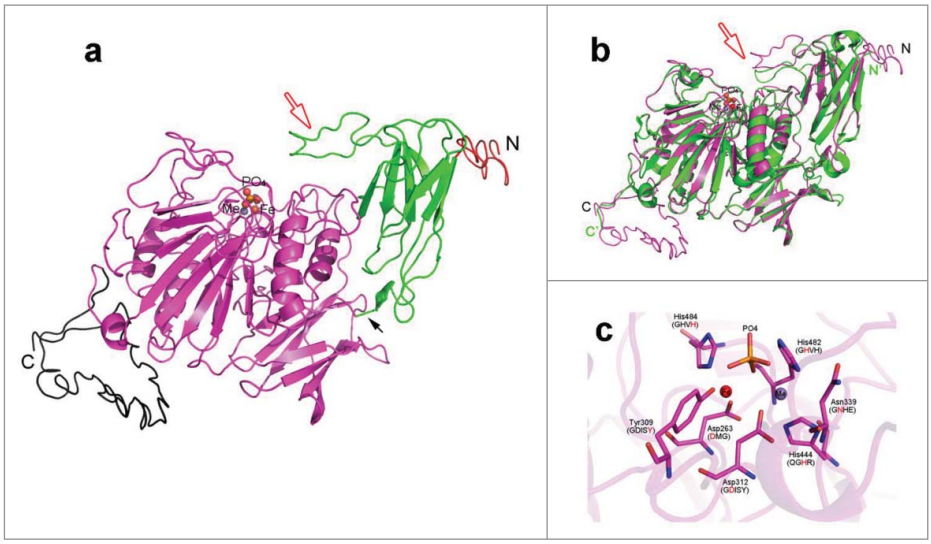

Figure 3. The predicted structure of AtPAP2. (A) Different domains of an AtPAP2 monomer unit are shown in different colors (Red: transit peptide; Green: FNIII-like domain; Purple: PAP catalytic domain; Black: C-terminal hydrophobic motif; Blue sphere: Me (Zn or Mn) atom; Red sphere: Fe atom; Orange spheres: PO_4). A white arrow indicates the flexible loop. The hinge connecting the FNIII-like domain and the PAP catalytic domain is indicated by a black arrow. (B) The alignment of AtPAP2 (purple) and PPD1 (green) is shown as a cartoon highlighting their N- and C-termini. The metal atoms and phosphate ligands are shown as spheres. The loops connecting strands 3 and 4 of the FNIII-like domain are significantly different between AtPAP2 and PPD1 (white arrow). (C) The active site and bound phosphate of AtPAP2. The Fe and Me atoms are shown as red and blue spheres, respectively. The phosphate ligands are shown as sticks (red and yellow). The Fe atom is coordinated by protein residues His484, Tyr309, Asp263, and Asp306. The Me atom is coordinated by His444, His482, Asn339, and Asp306, which bridge both of the metal atoms. All of the metal chelating residues are shown as sticks, with labeling of the 5 conserved PAPs motifs (DMG, GDISY, GNHE, QGHR, and GHVH).

incubation for 48 hours in the dark, the infected regions of the leaves were detected for fluorescence under Confocal Laser Scanning Microscopy 710 NLO. As shown in Fig. 1B, AtPAP2 interacted with pSSU and mSSU but not the transit peptide in chloroplasts. AtPAP2 was shown to interact with pMORF3 in mitochondria,[3] but no signal in the mitochondria was seen for pSSU/mSSU, indicating that the interactions of AtPAP2 with pSSU/mSSU are specific to chloroplasts.

AtPAP2 modulates the import rate of [^{35}S]-labeled AtSSU1B into chloroplasts

AtSSU1B was chosen for chloroplast import study. The pSSU from tobacco has been shown to be phosphorylated at serine residue 34 on its transit peptide by STY kinases and leaf extract of *Arabidopsis thaliana*.[4] By homology analysis, we predicted the Ser32 residue on the transit peptide of AtSSU1B could be a phosphorylation site. The coding sequences of STY8 kinase (At2g17000), AtSSU1B (pSSU), mature AtSSU1B (mSSU), and a pSSU1B-S32A (pSSUm) mutant were subcloned into the pET28a vector (Novagen, Germany). Recombinant proteins were overexpressed in *E. coli* and purified by His-tag affinity column. *In vitro* Phosphorylation Assay was carried out as described.[4] Our data showed that recombinant AtSSU1B can be phosphorylated by STY8 kinase at Ser32 of its transit peptide (Fig. 2A-C). To prepare a highly purified chloroplast fraction that is free of cytosolic phosphatases, a protoplast isolation protocol was adopted.[14] Protoplasts were isolated from 14-day-old *Arabidopsis* seedlings harvested at the end of the night. The protoplasts were lysed by passing them through 10μm nylon mesh, and then gently layered over a discontinuous gradient of ice-cold 40% (v/v) Percoll (top) and 85% (v/v) Percoll (bottom) and centrifuged at 2,000 × g, 4°C for 10mins using a swing-bucket rotor. Intact chloroplasts collected from the top of 85% (v/v) Percoll were washed with HMS buffer (330mM sorbitol, 50mM HEPES/KOH (pH 7.6), and 3mM $MgSO_4$) and used for import assay. The TNT®-Coupled Wheat Germ Extract System (Promega) was used for the *in vitro* translation of [^{35}S]-labeled AtSSU1B. The kinase in Wheat Germ Extract (WGE) were shown to phosphorylate AtSSU1B (Fig. 2D). To complete the import process, the phosphorylated AtSSU1B must be dephosphorylated by a phosphatase prior to import. In this study, [^{35}S]-labeled AtSSU1B translated in WGE was imported into chloroplasts isolated from WT, *pap2*, and OE7 plants to compare the import efficiency.[15] We found that the import of WGE-synthesized [^{35}S]-AtSSU1B was more efficient in OE chloroplasts and less efficient in *pap2* chloroplasts, compared to the wild type (Fig. 2E-F). These data indicated that the dephosphorylation of the transit peptide of pSSU is required prior to importation into chloroplasts and that AtPAP2 anchored to the outer membrane of chloroplasts is a phosphatase responsible for this process.

Conclusion and perspective

Proteins in the cytosol enter chloroplasts and mitochondria through 2 similar but distinct protein channel complexes on the outer membranes-TOC and TOM. Both of these complexes contain channel proteins (Toc75/Tom40) and distinct receptors (Toc33/34, Toc159, and Toc64 on chloroplasts, and Tom20 and OM64 on mitochondria). A number of preproteins targeted to chloroplasts and mitochondria can be phosphorylated at their transit peptides/presequences.[3-5] To import pSSU and pMORF3 in their complex forms through the TOC and TOM, respectively, 2 additional steps are required: First pSSU/

pMORF3 must be dissociated from 14-3-3 and expose their transit peptide/presequence, followed by the dephosphorylation of the transit peptide/presequence. For the pSSU/14-3-3/Hsp70 complex, after its binding to Toc34, the hydrolysis of GTP to GDP at Toc34 may displace 14-3-3 and expose the phosphorylated residue so that it can be dephosphorylated by phosphatases. We propose that AtPAP2 is one of the phosphatases that exert this role. However, we failed to express enzymatically active recombinant AtPAP2 in *E. coli* or yeast and hence dephosphorylation assay could not be performed. The phosphorylation status of the transit peptide does not affect the specificity of organelle targeting but it does affect the rate of import.[16,17] For example, the import rate of phosphomimic S35-37D Hcf136-mSSU into chloroplasts was retarded.[17] After dephosphorylation, the transit peptide then binds to Toc159. The hydrolysis of GTP on Toc159 then pushes the preprotein through the translocation channel, Toc75.[8]

Similar to Toc34 and Tom20, AtPAP2 is anchored to the outer membranes of chloroplasts and mitochondria via its C-terminal transmembrane motif.[12] Both Toc34 and Toc64 bind to the transit peptide of pSSU but not to mSSU, whereas AtPAP2 binds to mSSU but not its transit peptide (Fig. 1A). Therefore, AtPAP2 may serve as a co-receptor for pSSU during its import into chloroplasts through the TOC complex. AtPAP2 is also present on the outer membrane of mitochondria. However, the interaction of AtPAP2 on mitochondria with these chloroplast-targeted proteins was not observed in the BiFC experiment (Fig. 1B), indicating that the presence of AtPAP2 is not sufficient for mitochondria to capture pSSU. For example, there is no Toc34 on the outer membrane of mitochondria, and hence the pSSU/14-3-3/Hsp70 complex could not bind to mitochondria. The import rate of the phosphorylated pSSU synthesized in WGE into *pap2* chloroplasts was reduced by ~25% (Fig. 2E). It is not clear if 100% WGE-synthesized pSSU are phosphorylated and if non-phosphorylated pSSU should be imported into the chloroplasts of all 3 lines (WT, OE7, *pap2*) at an equal rate. WGE contains high phosphatase activities.[3] We cannot exclude the possibility that some uncharacterized phosphatases in WGE may play a redundant role in dephosphorylating pSSU.

The full-length amino acid sequence of AtPAP2 was used to search the protein data bank at NCBI using BLASTp. A 3D model of AtPAP2 was built based on the X-ray structure of the best fitting structure (3ZK4) *Lupines luteus* purple acid phosphatase PPD1 by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) and the 3D model was edited by PyMOL v1.3r1 (Fig. 3A). Both PAPs belong to the large purple acid phosphatase family and contain an extra fibronectin type III-like (FNIII-like) domain at the N-terminus of the PAP catalytic domain (Fig. 3B). PPD1 exhibits DNase activity, and the FNIII-like domain of PPD1 was postulated to position the DNA substrate relative to the catalytic domain.[18] A 26-residue loop (Phe59-Pro85) of PPD1 connecting strands 3 and 4 extends approximately 20 Å from the barrel, and the Lys74 side chain is only 10 Å from the dimetal catalytic site of the PAP domain. This loop is stabilized by a disulfide bond (Cys69-Cys82) in PPD1. The model of AtPAP2 shows that this protein is highly homologous to PPD1, except for this loop and the additional C-tail that anchors AtPAP2 to the outer membranes of organelles. AtPAP2 also carries a loop connecting strands 3 and 4 of the β-sheets, but the amino acid sequence is completely different from that of PPD1 and does not contain any disulfide bond. Nonetheless, the loop of AtPAP2 is still in close proximity to the catalytic site of PAP. It is possible that the FNIII-like domain of AtPAP2 assists the PAP catalytic domain in capturing the mature portion of precursor proteins and allows the PAP catalytic domain to dephosphorylate the transit peptides. The FNIII-like domain is only connected to the PAP catalytic domain by a hinge, which could offer great conformational flexibility for AtPAP2 to bind to various interacting proteins.

AtPAP2 is the only phosphatase that is dually-targeted to the outer membranes of both chloroplasts and mitochondria. Overexpression of native, dual-targeting AtPAP2 in *Arabidopsis* enhanced sucrose and ATP production in leaves. By contrast, overexpression of AtPAP2 solely targeted to the outer membrane of mitochondria created transgenic plants with lower sucrose levels but higher ATP levels in leaves. AtPAP2 thus plays an important role in the carbon homeostasis of leaf cells by modulating the activities of chloroplasts and mitochondria. In addition to higher plants, homologs of STY kinase and PAP with a C-terminal hydrophobic motif can also be found in the genomes of the smallest free-living photosynthetic green algae, including *O. tauri* and *M. pusilla*. Each of these algal cells contains one chloroplast and one mitochondrion. The homeostatic regulation of carbon production by chloroplasts and carbon consumption by mitochondria is particularly important to unicellular photosynthetic cells. Modulation of protein import into these 2 organelles by phosphorylation and dephosphorylation of transit peptides/presequences may be an important regulatory process of carbon homeostasis evolved in the green lineage.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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