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SHORT COMMUNICATION

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# Sequence and biochemical analysis of Arabidopsis SP1 protein, a regulator of organelle biogenesis

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**ABSTRACT**
Peroxisomes, chloroplasts, and mitochondria are essential eukaryotic organelles that host a suite of metabolic processes crucial to energy metabolism and development. Regulatory mechanisms of the dynamics and biogenesis of these important organelles have begun to be discovered in plants. We recently showed that, aside from its previously reported role in targeting chloroplast protein import proteins, the Arabidopsis ubiquitin E3 ligase SP1 (suppressor of ppi1 locus1) negatively regulates peroxisome matrix protein import by promoting the ubiquitination and destabilization of PEX13 and possibly PEX14 and other components of the peroxisome protein import apparatus. Here, we compared protein sequence and domain structure of SP1-like proteins in Arabidopsis and their human homolog, Mitochondrial-Anchored Protein Ligase (MAPL). We further characterized SP1 protein in respect to its membrane topology and ubiquitin E3 ligase activity.

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Eukaryotic cells contain various membrane-delimited organelles that host specific sets of biochemical reactions. In plants, peroxisomes, mitochondria and chloroplasts are critical for various aspects of plant physiology, especially energy metabolism. Mitochondria and chloroplasts are surrounded by double membranes, while peroxisomes are single membrane-bounded. Plant peroxisomes are important for many physiological processes, including lipid mobilization, photorespiration, detoxification, hormone biosynthesis and plant-pathogen interaction.[1,2] Some of these functions, such as photorespiration, fatty acid metabolism and jasmonic acid biosynthesis, are accomplished coordinately by peroxisomes, mitochondria and/or chloroplasts.[2-4] To adapt to developmental and environmental changes, these organelles can adjust their abundance, distribution, morphology and biochemical activities. Core protein machineries governing key aspects of organelle assembly, division and protein import have been identified.[2,5-8] However, how the activities of these core components are regulated in response to various stimuli remains largely unknown. Dissecting the regulatory mechanisms of energy organelle dynamics is essential to answering the question of how plants adjust their energy metabolism to meet developmental and environmental requirements.

In Arabidopsis, the abundance and morphology of peroxisomes are affected by stress conditions such as high light, salt, pathogen and cadmium.[9-14] Transcriptional events that modulate peroxisome fission in response to light have been identified.[13-15] At the post-transcriptional level, a signature lipid of mitochondrial membrane, cardiolipin, plays a positive role in mitochondrial fission by supporting the oligomerization of dynamin-related protein 3A (DRP3A) and DRP3B, major fission proteins shared by mitochondria and peroxisomes in Arabidopsis.[16] In addition, the mitochondrial membrane-localized ubiquitin-specific protease UBP27 — the first ubiquitin-related enzyme reported to be associated with plant mitochondria — contributes to mitochondrial morphogenesis possibly by promoting the translocation of DRP3 from mitochondria to the cytosol.[17] More recently, we showed that the ubiquitin E3 ligase SP1 regulates peroxisome biogenesis via the ubiquitin-proteasome system (UPS) by promoting the ubiquitination and destabilization of PEX13 and possibly PEX14 and other components of the matrix protein import machinery.[18] SP1 had also been shown previously to play a similar function in chloroplast by targeting chloroplast protein import factors such as TOC33 for destabilization.[19]

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SP1 has 2 homologous proteins in Arabidopsis, SPL1 (SP1-Like 1) and SPL2, and a homologous protein in human named Mitochondrial-Anchored Protein Ligase (MAPL).[20] SP1, SPL1 and SPL2 are all associated with chloroplasts,[19] and have stable (SP1), weak/partial (SPL1), or no (SPL2) peroxisome localization.[18] Human MAPL localizes to both mitochondria and peroxisomes.[21,22] These findings suggest that this family of E3 ligases may be involved in the function of multiple organelles important for energy metabolism across diverse species. Arabidopsis SP1, SPL1, SPL2 and human MAPL share significant degrees of sequence similarity and similar predicted protein structure (Fig. 1A). Our phylogenetic analysis grouped SP1 and SPL1 in a separate subfamily from that of SPL2 (Fig. 1B), and both subfamilies appear to be conserved

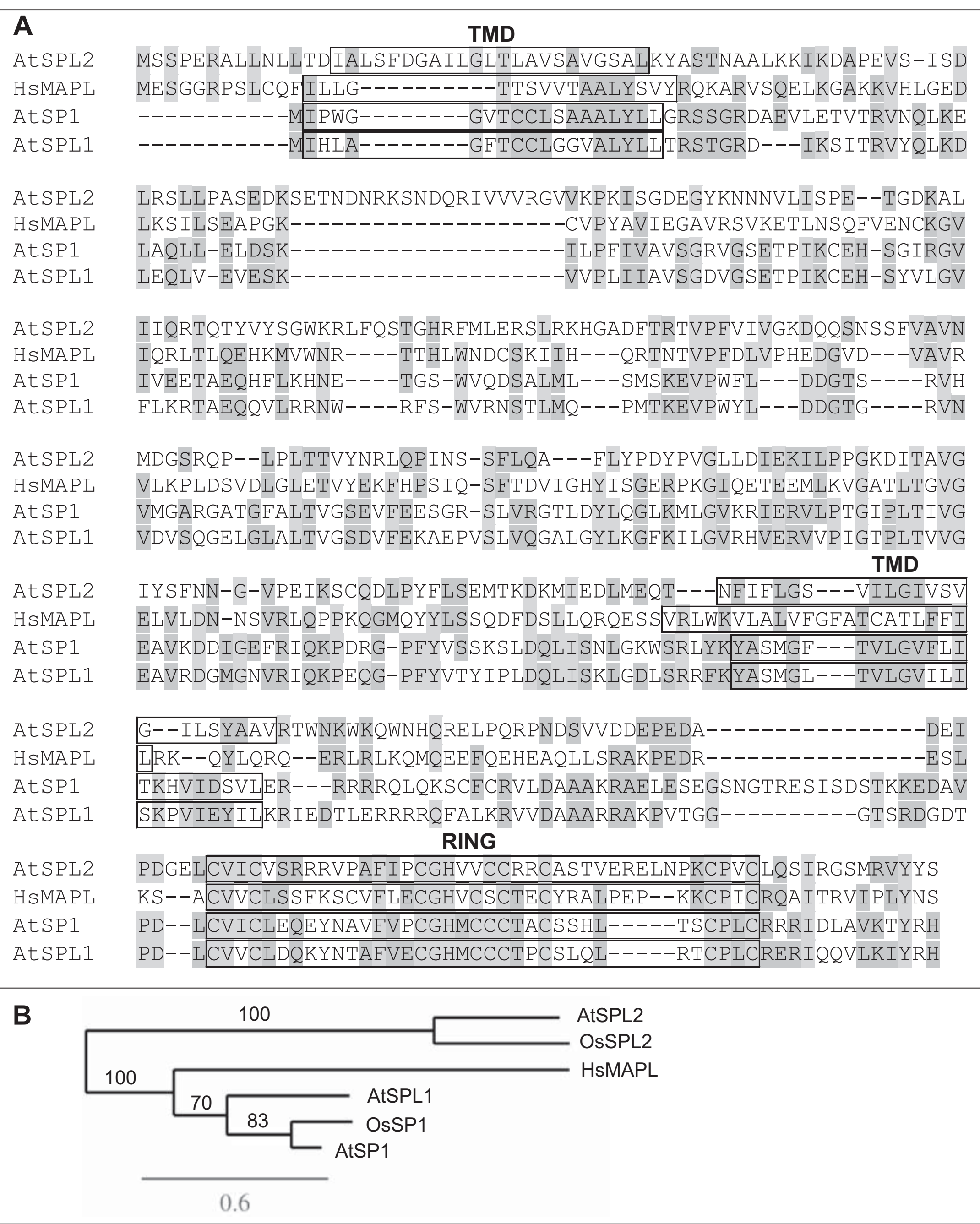


**Figure 1.** Sequence analysis of Arabidopsis SP1, SPL1 and SPL2 and human MAPL proteins. (A) Amino acid sequence alignment of SP1, SPL1, SPL2 and MAPL performed by the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical and similar residues are shaded. Predicted transmembrane (TMD) and RING domains are indicated by boxes. (B) Phylogenetic analysis of plant SP1-related proteins and human MAPL by Phylogeny.fr (http://www.phylogeny.fr/). At, *Arabidopsis thaliana*. Os, *Oryza sativa*. Hs, *Homo sapiens*. Scale bar, 0.6 amino acid substitutions per site. Branch support values are shown as percentage.

across plant species.[18] Human MAPL is more closely related to SP1 and SPL1 than to SPL2 (Fig. 1B), consistent with previous discoveries that SP1, SPL1 and MAPL, but not SPL2, are associated with the peroxisome to various degrees.[18,23]

Previous studies using the online plant membrane protein database Aramemnon (http://aramemnon.uni-koeln.de/) predicted that SP1, SPL1 and SPL2 each contain 2 transmembrane domains (TMDs).[18,19] However, when we subjected these proteins to TMD probability analysis using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) that predicts transmembrane helices in proteins, the probabilities for the N-terminal TMDs in SP1 and SPL1 were much lower than the others (Fig. 2A), indicating that SP1 and SPL1 may only contain a C-terminal TMD. Our previous study demonstrated SP1 to be an integral peroxisomal membrane protein,[18] and SP1 was reported to localize to the chloroplast outer envelope with the C-terminus facing the cytosol.[19] However, using protease protection assays with thermolysin, which degrades proteins unprotected by the organelle membrane, we found a more complex topology of SP1 on the peroxisome membrane. Whereas the peroxisome membrane protein PEX14 was totally degraded by thermolysin, only a portion of SP1-YFP was digested by the protease (Fig. 2B), indicating that SP1 may have more

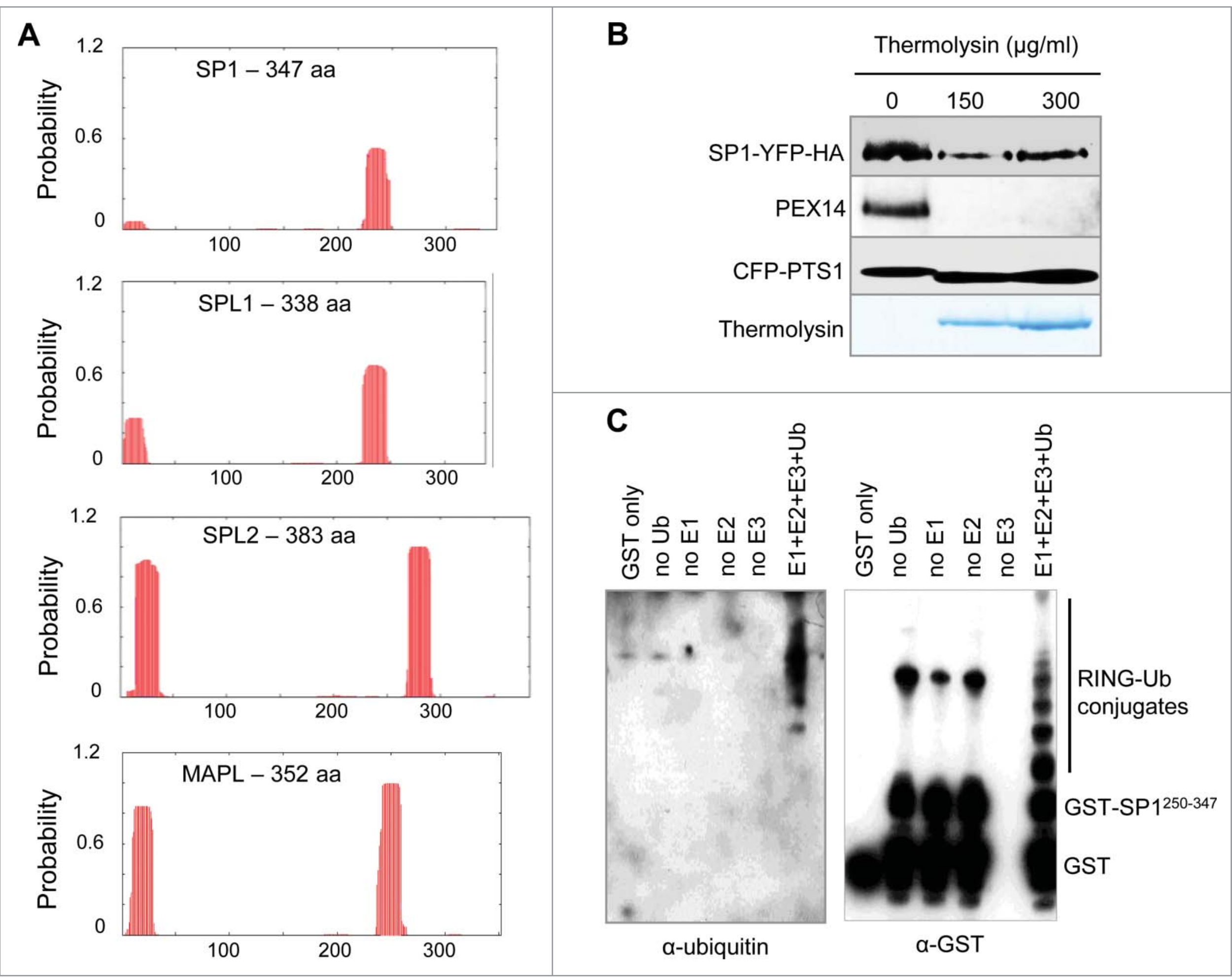


**Figure 2.** Characterization of the SP1 protein. (A) Transmembrane domain (TMD) analysis of Arabidopsis SP1, SPL1, SPL2 and human MAPL by the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). Columns indicate potential TMDs. Y axis represents the probability of TMDs, and x-axis indicates the length of the analyzed proteins. (B) Protease protection assay to determine membrane topology of SP1 on the peroxisome. Peroxisomes were isolated from Arabidopsis plants co-expressing SP1-YFP and CFP-PTS1 (peroxisome targeting signal type 1; SKL), which had been generated in our previous study,[18] treated with thermolysin, and subjected to immunoblot analysis with $\alpha$-GFP and $\alpha$-PEX14 antibodies, using protocols that we used previously.[29] Here, 200 $\mu$l purified peroxisomes was treated respectively with 0, 150 or 300 $\mu$g/ml of thermolysin in an incubation buffer containing 50 mM Hepes/NaOH, pH 7.5, 0.33 M sorbitol, and 0.5 mM $CaCl_2$. Reactions were performed at 4°C for 30 min, and stopped by 5-min incubation on ice with 5 mM EDTA, followed by immunoblot analysis. The thermolysin bands were Coommassie Blue-stained. (C) *In vitro* ubiquitination assays using a previously published protocol.[25] Immunoblot analyses were performed using anti-Ubiquitin (1:10,000; Invitrogen) and anti-GST (1:150; Sigma-Aldrich) antibodies.

than one form of topology on the peroxisome membrane. It is possible that a portion of peroxisomal SP1 have their C-terminus exposed to the cytosol whereas other SP1 proteins are exposed only to the peroxisome matrix side and therefore protected by the peroxisome membrane from thermolysin. However, we cannot exclude the possibility that the YFP tag affects SP1's membrane topology, or that the thermolysin resistant SP1 proteins were resulted from incorrect protein folding/assembly. A SP1 specific antibody that detects endogenous SP1 proteins may help to clarify this issue.

Multiple studies reported human MAPL to be an ubiquitin E3 ligase and a SUMO E3 ligase.[20,26] Here we tested SP1's ubiquitin ligase activity using an *in vitro* ubiquitination assay. A fusion protein of GST and the C-terminal 98 amino acids of SP1 that contains the RING domain (GST-$SP1^{250-347}$) was expressed and purified, and a cellular ubiquitination cascade was reconstituted using 6xHis-tagged ubiquitin, wheat E1, human UBCH5b (E2), and GST-tagged $SP1^{250-347}$ (E3). SP1 exhibited auto-polyubiquitination activity *in vitro* in the presence of E1, E2 and ubiquitin, whereas no activity was detectable in the absence of E1, E2 or E3 (Fig. 2C). This result confirmed results from previous reports, which used different assay systems to show that Arabidopsis SP1 possesses ubiquitin E3 ligase activity.[19,24]

With respect to the SUMO ligase activity of human MAPL, human Dynamin-Related Protein 1 (hDRP1), the core protein in mitochondrial and peroxisome fission and the human equivalent of Arabidopsis DRP3, was shown to be a substrate for MAPL-mediated SUMOylation.[20] It is possible that MAPL contains one or both activities when targeting different substrates, which led to the question of whether Arabidopsis SP1, SPL1 and SPL2 also have SUMO E3 ligase activities. Human SUMO1 (hSUMO1) localizes to mitochondria in a pattern similar to hDRP1, and interacts with hDRP1 in yeast 2-hybrid assays.[27] Arabidopsis contains 4 functional SUMOs: SUMO1, 2, 3 and 5.[28] Although we did not observe obvious organelle division defects in *sp1* mutants,[18] it may be worthwhile to check in the future whether Arabidopsis SUMO proteins also target the organelle division factor DRP3.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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