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Keep the balloon deflated

The significance of protein maturation for thylakoid flattening

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Thylakoidal processing peptidase (TPP) catalyzes the removal of signal peptide which leads to maturation of a subset of proteins including photosynthetic electron transport components in thylakoids. The biochemical properties of TPP were highly defined during the 1980's and 1990's, but the physiological significance of the TPP activity had remained undefined. Completion of genome sequencing revealed the presence of three TPP isoforms in the model plant *Arabidopsis thaliana*. A recent genetic study demonstrated that one isoform, plastidic type I signal peptidase 1 (Plsp1), is necessary for proper thylakoid assembly. Interestingly, Plsp1 was found in both the chloroplast envelope and thylakoids, being responsible for maturation of an outer membrane protein Toc75 and a lumenal protein OE33. A more recent study has shown that Plsp1 is involved in maturation of two additional lumenal proteins, OE23 and plastocyanin, and that accumulation of unprocessed Toc75 does not disrupt normal chloroplast development. The study also revealed that *plsp1*-null plastids accumulate balloon-like vesicles that appear to be the remnants of thylakoids as they contain unprocessed OE33 in the peripheral regions. These findings suggest that proper maturation of lumenal proteins is required for correct assembly and/or maintenance of thylakoids, but may not be necessary for initiation of membrane development. The ballooned thylakoids in *plsp1*-null plastids may be a useful tool to elucidate the mechanism of thylakoid flattening, which correlates with the energized state of the membranes.

Key words: *Arabidopsis thaliana*, attractive forces, chloroplasts, OE33, Plsp1, protein maturation, thylakoid lumen, thylakoidal processing peptidase, Toc75, type I signal peptidase

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Thylakoid lumen is an important compartment for oxygenic photosynthesis. Its compressed nature (**Fig. 1**) reflects proper functionality of the electron transport machinery.[1,2] All the known proteins found in the thylakoid lumen, including plastocyanin and subunits of oxygen evolving complex (OEC), OE33, OE23 and OE17, are encoded in the nucleus as a precursor form.[3] They are synthesized in the cytoplasmic ribosomes with a bipartite targeting sequence in their N-termini. The most N-terminal portion acts as a stroma-targeting sequence and is cleaved by the stromal processing peptidase (SPP), whereas the second sequence is required for further protein routing to the thylakoid and is removed by the thylakoidal processing peptidase (TPP) in the lumen. Identification and characterization of TPP activity were done by a series of biochemical studies in late 1980's to early 1990's.[4-8] Various fractionation techniques were used to show the integral association of TPP to non-appressed (but still flattened) lamellae of thylakoids with the active site on the lumenal face.[9] Analysis of its reaction specificity revealed that TPP belongs to the type I signal peptidase family,[10,11] a group of integral membrane proteins cleaving signal peptides from preproteins at various membrane systems including the plasma membrane of prokaryotes and the endoplasmic reticulum and mitochondria inner membrane in eukaryotes.[12] TPP substrates include not only the lumenal proteins, but also a subset of proteins integrally associated to the thylakoid membrane.[3]

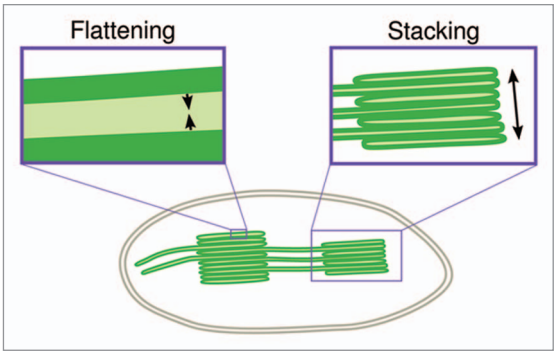

Figure 1. Thylakoid flattening and stacking. Flattening refers to the narrowing of the lumenal space by attraction of two opposing inner surfaces of the thylakoid membrane. This attraction is presumed to be facilitated by acidification of the thylakoid lumen in high light and to a lesser extent by Mg^{2+} in the dark.[1] Conversely, stacking is the organization of thylakoids into grana, which requires stabilization between the outer surfaces of adjacent membranes. This stabilization is attributed to phosphorylation status of light harvesting chlorophyll a/b binding proteins, LHCIIs.[2]

Although it is generally believed that complete removal of a signal sequence reveals the active protein,[12] only a handful of studies have demonstrated its significance. Indeed, a few reports have shown that improper maturation of TPP substrates has little to no disruptive effect. For example, an unprocessed form of the membrane-bound cytochrome f, the chloroplast-encoded TPP substrate, was shown to be assembled correctly into the functional cytochrome b_6f complex in *Chlamydomonas reinhardtii* thylakoids in vivo.[13] In another report, unprocessed OE33 from spinach was still able to maintain O_2 evolution at the level near the fully processed form when produced in bacteria and reconstituted in the membrane fraction containing the photosystem II core components.[14]

In 1998, the first TPP cDNA sequence of higher plants was reported from *Arabidopsis thaliana* based on the sequence similarity of the encoded protein to a cyanobacterial enzyme.[15] Completion of the genome sequencing revealed the presence of two additional TPP isoforms in this model species.[16-18] One of them, named plastidic type I signal peptidase 1 (Plsp1), was re-discovered by a genetic screen for a component responsible for the complete maturation of the protein translocation channel Toc75 (for translocon at the outer-envelope-membrane of chloroplasts 75).[19] Interestingly, knockout of the *PLSP1* gene expression results in a severe disruption of thylakoid development and seedling lethality, which coincides with the accumulation of unprocessed forms of not only Toc75, but also a lumenal protein, OE33.[19] A subsequent cytological study demonstrated that Plsp1 is located in both the envelope and thylakoids, and its localization pattern corresponds to thylakoid development, supporting the notion that Plsp1 is physically involved in maturation of both Toc75 and OE33.[20] However, it had remained elusive if complete maturation of Toc75 or that of OE33, or both, is necessary for proper thylakoid development. Furthermore, the suborganellar localization of the unprocessed OE33 in the "grana-less" plastids in *plsp1*-null plants had been unknown.

Recently, we conducted an extensive study to address the mechanism by which Plsp1 plays a role in thylakoid development.[21] The comprehensive analysis revealed that two additional TPP substrates, OE23 and plastocyanin, but not OE17, accumulate as unprocessed forms in *plsp1*-null plastids. A transmission electron microscopy study of the mutant plants demonstrated that, while plastids in young leaves are capable of initiating a partly flattened internal membrane system, those in fully-unfolded leaves no longer maintain this organization. Instead, plastids in mature leaves of the mutants, even grown under low light conditions, contain only a disjointed system of stroma-localized "balloon-like" vesicle bodies, which contain unprocessed OE33 at the peripheral area (**Fig. 2**). Furthermore, a genetic complementation assay revealed that accumulation of improperly-processed Toc75 does not disrupt normal chloroplast development. Together, these data suggest that Plsp1 may be the main TPP in *A. thaliana* chloroplasts, although the proper maturation of OE17 could depend on the activity of one of other TPP isoforms. These data also indicate that complete maturation of lumenal proteins may be necessary to ensure the proper assembly of thylakoids.

What can we learn from the balloon-like vesicles in *plsp1*-null plastids? Similar structures are often observed in mutant plastids exhibiting a severe disruption of thylakoids,[22-24] although their properties remain undefined. In *plsp1*-null plastids, the presence of unprocessed OE33 (**Fig. 2**) indicates that such vesicles may be the remnants of thylakoid membranes containing the functional cpSec translocon, which is responsible for ATP-dependent OE33 translocation. In addition, slight but significant accumulation of unprocessed OE23 suggests the presence of another lumenal protein-targeting pathway, the cpTat pathway that depends on proton motive force.[25] If this is the case, how is the energy driving the protein targeting provided in the apparent non-photosynthetic plastids? A hint to answer this question may come from a previous study demonstrating the presence of both cpSec and cpTat pathways in the internal membranes of chromoplasts in red bell pepper fruits.[26] The functionality of the cpSec transport was supported by the presence of the stromal compartment cpSecA, whereas F_oF_1 ATP synthase in the internal membrane was postulated to provide the energy for the cpTat pathway by hydrolyzing ATP in the absence of the photosynthetic electron transport to generate membrane potential.[26] Similarly, *plsp1*-null plastids may contain cpSecA in the stroma, and the ATP synthase may still be present in the unflattened thyakoids, contributing to the generation of required membrane potential. In this scenario, the presence of photosynthetic electron transport is not essential for cpSec and cpTat pathways.

The ballooned thylakoids (**Fig. 2**) may also be a useful tool to test the mechanism of thylakoid flattening, which is proposed to be enhanced by lumenal attractive forces contributed partly by $[H^+]$ generated by light-driven electron transport.[1] In

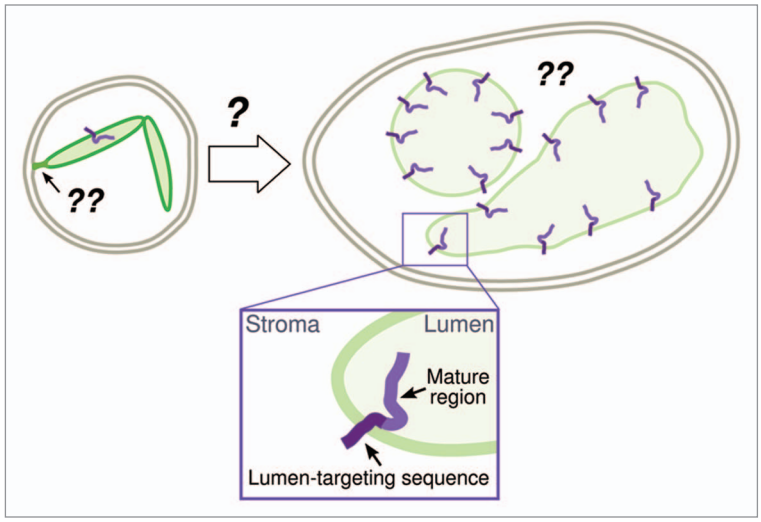

Figure 2. Thylakoid inflating in *plsp1*-null plastids.[21] Plastids in newly-developed true leaves of *plsp1*-null plants (left) have partly flattened internal membrane systems, which appear to be "premature" thylakoids and may be continuous to the inner envelope membrane (denoted by a black arrow). The accumulation of unprocessed lumenal proteins such as OE33 may lead to expansion of the lumen space and the resulting balloon-like vesicles in plastids of older leaves (right). The unprocessed lumenal proteins may associate with the membranes via the hydrophobic region in their N-terminal lumen-targeting sequence, facing the mature regions within the lumen as shown in the expanded box.

plsp1-null plastids, unprocessed OEC subunits and plastocyanin may be stuck in the translocons or lipid bilayers as discussed,[21] and thus may not be incorporated into the functional complex. This would lead to the disruption of electron transport chain and hence result in the loss of lumenal attractive forces, which are necessary to keep the thylakoid inner surfaces in close contact. Alternatively, accumulation of the unprocessed lumenal proteins may somehow directly enhance the repulsive forces, e.g., the negative charges of the membrane surfaces.[1] Detailed characterizations of the membrane vesicles from the *plsp1*-null plastids will be necessary to address these possibilities.

In summary, the close examination of the *plsp1*-null plants has shined new light on the old enzyme TPP, revealing the novel level of complexity in thylakoid assembly. The ballooned thylakoids found in *plsp1*-null plastids may be a useful tool to address questions relevant to assembly of thylakoids and other membrane systems in general.

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