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Thylakoid Lumen; from "proton bag" to photosynthetic functionally important compartment

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This mini review provides an update of the thylakoid lumen, shedding light on its intricate structure, unique proteome, and potential physiological significance. This compartment within the thylakoid membranes of chloroplasts was originally perceived as "empty", only providing a site for proton accumulation to support ATP formation. Instead, recent investigations have revealed that the lumen houses a specific set of proteins each with potentially critical roles. The structure of this compartment has been shown to be dynamic, with changes in size and organization influenced by light exposure, impacting protein mobility and function. Noteworthy, some of the lumen proteins are permanently or transiently in contact with protein complexes located in the thylakoid membrane, such as PSII (PsbP-like and PsbQ-like proteins) cytochrome b_6f, and PSI. Meanwhile, other lumen proteins seems to be more "independent" such as proteases, immunophilins, stress-related proteins, pentapeptide repeat proteins, and many others with unknown functions. All these proteins play crucial roles in maintaining photosynthetic machinery, adapting to environmental stress, and regulating cellular processes. Understanding the lumen's function is vital as it holds promise for uncovering novel regulatory interactions and signaling pathways within the chloroplast.

KEYWORDS

photosystem II, proteases, immunophillins, water oxidation, grana

1 Introduction

The process of photosynthesis, in which the solar energy is converted to bound chemical energy in the form of starch, is a specific characteristic of plants, algae, and cyanobacteria. In plants, this process takes place in a specific organelle, the chloroplast. The chloroplast has a double membrane, the envelope, which substantiates its bacterial origin. Inside the chloroplast, the thylakoid membranes are located, hosting Photosystems I and II (PSI and PSII), the cytochrome b_6f complex, and ATP-synthase supramolecular complexes. The thylakoid membrane has a fascinating architecture, differentiated into cylindrical grana

stacks and tubular stroma lamellae. Nestled within the thylakoid membrane is the thylakoid lumen, which forms a continuous compartment with the thylakoid membrane at its outer border. This secluded compartment emerges as a microscopic realm of immense significance in the process of photosynthesis. In this mini review, we provide an update on the content of this previously considered "empty" chloroplast compartment.

The main function of the thylakoid lumen is accumulation of protons to support ATP formation via ATP-synthase and only a few proteins were suggested to be in this compartment: two extrinsic PSII proteins (PsbP and PsbQ) and plastocyanin, the electron linker between PSII and PSI. Thus, many textbooks show an empty lumen space with protons (H^+) inside. It has been 25 years since the first attempts to obtain a detailed general analysis of the lumen content was published (Kieselbach et al., 1998a). This paved the way for a more detailed proteomic analysis of this compartment until recent days (Kieselbach et al., 2000; Bricker et al., 2001; Peltier et al., 2002; Schubert et al., 2002; Järvi et al., 2013; Gollan et al., 2021). It is now well-established that the thylakoid lumen has a specific proteome (Table 1) that is distinct and separate from the stroma and cytoplasm compartments, containing several unique proteins and protein groups. The protein concentration in the lumen has been estimated to be 20 mg of proteins per mL, resulting in a protein concentration of the more abundant lumen proteins in the micro to millimolar range, which is similar to that of many stroma proteins (Kieselbach et al., 1998a). Interestingly, already in 1988 (Weibull and Alertsson, 1988), it was suggested that the lumen could contain proteins, as the space between the thylakoid membranes in electron microscopy studies was not transparent.

In the pioneer proteomic studies, the lumen protein content was estimated to be 80 (Schubert et al., 2002) or even up to 200 (Peltier et al., 2002) distinct proteins. In Table 1, we have, to the best of our knowledge, accumulated all the lumen protein identified or suggested in the literature so far. Table 1 contains 62 proteins that have been biochemically identified in various lumen extractions, and at least 16 further proteins have been predicted through various genome searches and the prediction of signal peptides and cleaving sites (Peltier et al., 2002; Schubert et al., 2002; Gollan et al., 2021), thus suggesting their import into the lumen. This makes a total of 78 lumen proteins at present time. Several of these lumen proteins have been found to have homologs in *Synechocystis* sp. PCC 6803 (see Table 4 in Kieselbach and Schröder, 2003). Of the 78 proteins, 43 have been both predicted and biochemically identified, giving stronger evidence for their location in the lumen. The functions of these proteins will be discussed in a separate section.

2 Structure and dynamics of the thylakoid lumen

The introduction of electron microscopy in the 1930s enabled a significant advancement from the "green dot" chloroplasts previously observed using light microscopy. More details could be seen, and new 3D structures of the thylakoid membrane were developed. A thorough description of the history of how microscopic studies have advanced the understanding of plant thylakoids can be found in (Staehelin and Paolillo, 2020). A typical thylakoid grana stack has a diameter of 300nm, containing 3-19 (average of 9) grana sacks with an average height of 160 nm (Bussi et al., 2019). In the helical fretwork model (Paolillo, 1970) each granum is surrounded by multiple stroma lamellae connected to the granum by slits at the rim of the grana stack (Mustárdy et al., 2008; Nevo et al., 2009; Daum et al., 2010). This model has been modified based on electron tomography studies resulting in the "bifurcation model", in which the stroma lamella is located perpendicularly to the grana and connected by slit-like apertures to the rim of the grana stacks (Figure 1 top). The connection points form a right-handed helical structure around the grana (Mustárdy et al., 2008; Daum et al., 2010; Austin and Staehelin, 2011). Recently, a mix of right and left-handed connection points between the grana and stroma membrane was suggested to reduce twisting tension (Bussi et al., 2019).

The original model of the thylakoid membrane had long extensions of the grana stacks forming stroma lamellae (see for instance Kieselbach and Schröder, 2003). This model implied a fast and free flow of proteins between the two membrane sections. However, the new structural findings with slit-like apertures between grana and stroma lamellae questions how freely proteins can move within the lumen compartment and/or between grana stroma regions. Restricted mobility between the two regions is supported by the finding that protein movement in the grana stack region is less than 20% compared to 50% in the stroma lamellae (Murphy, 1986; Kirchhoff et al., 2011; Kirchhoff et al., 2013). This could explain why some lumen-identified proteins are present in higher concentrations in grana, while others are more present in stroma fractions (Gollan et al., 2021). It must be kept in mind that most of the lumen-located proteins do not seem to form complexes, which would make their movement even more difficult.

The number of stacked membranes in the grana is dependent on the light conditions. At low light more grana are formed and the diameter of the grana stack increases (Anderson et al., 2012). There have also been publications suggesting that the width of the lumen in the grana can vary, especially between light-adapted and dark-adapted plants (Murakami and Packer, 1970; Kirchhoff et al., 2011). Recently, based on optimized fast sample fixation techniques for electron microscopy, it was possible to obtain numbers for the swelling of the lumen upon light exposure from Arabidopsis (Li et al., 2020). Li et al. (2020) showed that the swelling occurs both in the grana stack and in the stroma lamellae (Figure 1, lower part). The grana sack in the dark was found to be 12.2 nm high, meanwhile in the light this increased to 15.7 nm (Li et al., 2020). Assuming an average of 9 sacks in a granum stack, the dark grana stack will have a height of 170 nm height, while the light exposed photosynthesising grana stack will expand to 190 nm. If we assume that the thylakoid membrane is 4.0 nm on an average, then the inner lumen distance in the grana sack in light is 7.7 nm but only 4.2 nm in the dark (Figure 1, lower part). Meanwhile the diameter of the grana stack has been reported to be unchanged in different light conditions and also between plant species (Albertsson and Andreasson, 2004). These findings point toward some new ideas with respect to the thylakoid lumen; The light expansion of the lumen leads to a dilution

TABLE 1 Proteins found and or predicted to be located in thylakoid lumen of *Arabidopsis thaliana*.

Gene	Protein name	Mass (kDa)	Ref	I/P	*	Comment
Photosystem II associated						
AT5g23120/.1	Hcf136, H136_ARATH	35.9	1,3,4	I/P		PSII
AT1g51400	PsbTn2	11.4	3	I/P		PSII
AT1g03610	Psb27-1, 11.9kDa	11.9/11.7	2.7	I/P		PSII
AT5g66570	PSO1_ARATH	ND/26.6	1,2,3,4	I/P	MA	PSII
AT3g50820	PSO2_ARATH	24.7/26.6	1,2,3,4	I/P	MA	PSII
AT1g06680	PSP1_ARATH	ND/20.2	1,2,3,4	I/P	MA	PSII
AT2G30790	PSP2_ARATH		1	P		PSII
AT4g21280	PSQ1_ARATH	ND/16.3	1,2,3,4	I/P	MA	PSII
AT4g05180	PSQ2_ARATH	15.3/16.3	1,2,3,4	I/P	MA	PSII
PsbP domain/like						
AT3g55330	PPL1/TL26_ARATH	16.3/17.8	1,2,3,4	I/P		PSII repair but not associated to the complex
AT2g39470	PPL2/26.3kDa protein	23.6/18.6	1,2,3,4	I/P		lumenal NDH subunit
AT4g15510	PPD1/T215_ARATH	21.5/21.3	1,2,3,4	I/P		PSI assembly factor for proper folding and integration of PsaB and PsaA
AT2G28605	PPD2	14.7	3	I		
AT1g76450	PPD3/15.9-kDa protein	15.9/15.8	1,2,3,4	I/P		
AT1g77090	PPD4/TL30_ARATH	19.4/22.2	1,2,3,4	I/P		
AT5g11450	PPD5/35.8 kDa protein	24.3/25.6	1	I/P		
AT3g56650	PPD6/20-kDa protein	20.0/21.5	1	I/P		May be involved in the redox regulation of PSII
AT3g05410.2	PPD7	23.2	3	I		
At5g27390	PPD8	17.5	3.5	P		
AT3g63525.1	PPD9	20.8	1,2,3	I/P		
PsbQ domain						
AT1g14150	PQL1	17.4	1,3,4	I/P	MA	lumenal NDH subunit
AT3g01440	PQL2	13.8	1,3,4	I/P	MA	lumenal NDH subunit
AT2g01918	PQL3	14.1	3.7	I		Required for both formation and activity of NDH complex
Cytochrome c						
AT5g45040	Cyt c6a/CYC6_Arath	23.2/29.3	3	I		electron carrier between cyt b6/f and PSI
Plastocyanins						
AT1g76100	PLAS_ARATH	ND/10.5	1,2,3,4	I/P		Minor
AT1g20340	PLAT_ARATH 0	16.2/10.5	1,2,3,4	I/P		Major
Radical and stress related proteins						
AT4g09010/.1	TL29/ APX4/APX-like	23.2/29.3	1,2,3,4	I/P		
AT3g26060	PrxQ	16.7	8	I		peroxiredoxin

(Continued)

TABLE 1 Continued

Gene	Protein name	Mass (kDa)	Ref	I/P	*	Comment
AT1g08550	Violaxanthin de-epoxidase	44.2/39.8	1,2,3	I		Xanthophyll cycle
Proteases						
AT5g46390	CTPA1/D1-protease like	49.0/45.8	1,2, 3	I/P		Peptidase S41 family protein
AT4g17740	D1-processing protease	39.6/41.9	1,2,3	I/P		Tail-specific proteases
AT3g57680	CTPA3/P45.4	nd/43.9	3	I/P		Peptidase S41 family protein
AT3g27925	DegP1 protease	31.7/35.2	1,2,3,4	I/P		Serine proteases, trypsin family
AT4g18370	DegP5/HhoA protease	27.4/23.5	1,2,3,4	I		Serine proteases, trypsin family
AT5g39830	DegP8/36-kDa protein	31.3/37.5	1,2,3	I		Serine proteases, trypsin family
Immunophilins						
AT5g13120	CYP20-2/18.5-kDa	18.5/20.0	1,2,3,4	I		Cyclophilin-type PPIases
AT1g74070	CYP26-2/P22.2	26.2	3	I/P	MA	Cyclophilin-type PPIases
AT5g35100	CYP28/ 26kDa	25.5/27.9	1,2,3	I		Cyclophilin-type PPIases
AT3g15520	CYP37/38-kDa protein	38.0/37.3	1,2,3	I/P		Cyclophilin-type PPIases
AT3g01480	CYP38/40-kDa protein	36.1/38.2	1,2,3,4	I/P		Cyclophilin-type PPIases
AT5g45680	FKBP13/14.7-kDa protein	14.7/13.6	1,2,3	I		FKBP-type PPIases
At4g26555	FKBP16-1	15.5	3.5	I		FKBP-type PPIases
AT4g39710	FKB16-2/21.2kDa	21.2/15.5	2,3,4	I/P		lumenal NDH subunit
AT2g43560	FKBP16-3	17.5/15.7	1,2,3,4	I/P		FKBP-type PPIases
AT3g10060	FKBP16-4/P17.8	17.3	1,3,4	I/P	MA	FKBP-type PPIases
AT4g19830	FKBP17-1/P16.7	16.7	1.3	I/P		FKBP-type PPIases
AT1g18170	FKBP17-2/P16.5	16.9	3.7	P	MA	FKBP-type PPIases
AT1g20810	FKBP18	17.8/17.9	1,2,3	I/P		FKBP-type PPIases
AT5g13410	FKBOP19/18-kDa protein	18.0/18.7	1,2,3,4	I		FKBP-type PPIases
AT3g60370	FKKBP20-2/16.9-kDa	16.9/16.4	1,2,3	I/P		FKBP-type PPIases
Y12071	TLP40	49.9	9	I/P		PPIase and Has a regulatory effect on thylakoid protein phosphorylation
Pentapeptide proteins						
AT5g53490	TL17 PRP	15.4/17.4	1,2,3,4	I/P		
AT2g44920	TL15 PRP	14.7/11.5	1.6	I/P		
AT1g12250	TL20.3 PRP/P20.1	ND/20.3	6.7	I/P	MA	
Other Proteins						
AT5g52970	TL15 15.0-kDa protein	15.0/11.5	1,2,3,4	I/P		
AT4g24930	TL17 17.9-kDa protein	17.9/18.0	1,2,3,4	I/P		
AT1g54780	TL18 18.3-kDa protein	18.3/22.1	1	I/P		putative phosphatase
AT3g63540	TL19 19-kDa protein	19.0/ND	1	I		
AT4g02530	MPH2/TL16_ARATH	15.3/17.6	1,2,3,4	I		
AT3g47860	LCNP (CHL)	10.6	3	I	MA	Lipocalin, prevents thylakoidal membrane lipids peroxidation

(Continued)

TABLE 1 Continued

Gene	Protein name	Mass (kDa)	Ref	I/P	*	Comment
						and confers protection against oxidative stress
AT2g26340	P19.1	20.4	3	I/P	MA	
AT2g23670	YCF37-Like/P10	10	1,3,7	I/P	MA	
AT5g64040	PsaN	10.6	3.4	I	MA	Mediate the binding of the antenna complexes to the PSI reaction center and core antenna
AT5g42765	P18.3	18.3	7	I	MA	Plasma membrane fusion protein
AT2g34860	PSA2 PDI	11.9	3, 6	I		Nuclear genome-encoded factor required for the accumulation of PSI
AT2g36145	P12.3	12.3	3, 6	I		auxin response genes expression
AT3g03630	CS26	34.3	3	I		Cystein syntas 26, essential for light-dependent redox regulation and photosynthetic performance
AT2g40400	RER5 (P71)	80.7		P	MA	May play a role in leaf development
AT2g37400	P28.2	38.1		P		
AT3g56140	RER6 (P73.5)	82.3		P	MA	May play a role in leaf development
AT1g33780	P28.3	36.3		P		Electron trasporter putative
AT3g09490	P38.4	38.4		P		
AT3g44020	P17.1	17.1		P		
AT5g02590	P36.5	37.3		P	MA	Tetratricopeptide repeat (TPR)-like superfamily protein
AT1g79450	ALIS5 (P37.9)	38.9		P	MA	Required for the lipid transport activity of the ALA/ALIS P4-ATPase complex
AT1g51350	P65.8	72.4		P		ARM repeat superfamily protein
AT1g621140	P40.1			P		
AT1g14590	P38	44.4		P		
AT5g46560	P36.5	44.3		P		
AT1g21500	P13.2	7.6		P		
AT3g08550	P53.8	59.9		P		Glycosyltransferase
AT2g03420	6.2 kDa protein	19.0	2	P		

I: Identified/detected protein.
P: Predicted lumen protein.
1. Schubert et al. (2002).
2. Kieselbach and Schröder (2003).
3. Gollan et al. (2021).
4. Peltier et al. (2002).
5. Granlund et al (2009).
6. Friso et al. (2004).
7. Zybailov et al. (2008).
8. Hall et al. (2010).
9. Fulgosi et al. (1998).
*MA, Membrane-Attached, i.e. proteins that are permanently or transiently in contact with protein complexes located in the thylakoid membrane.

(40-50%) of the lumen content in the light compared to the dark. Further investigation is required to determine if such dilution would influence or change the enzymatic activity of enzymes located in the lumen. An additional point of interest is that cryo-EM analysis shows that the PSII complex extends into the lumen by 5 nm (Graça et al., 2021), which raises the question, what happens to the extrinsic part of PSII when the space "shrinks" to only 4.2 nm in the dark (see Figure 1)? Are the extrinsic proteins PsbO, PsbP and PsbQ removed and

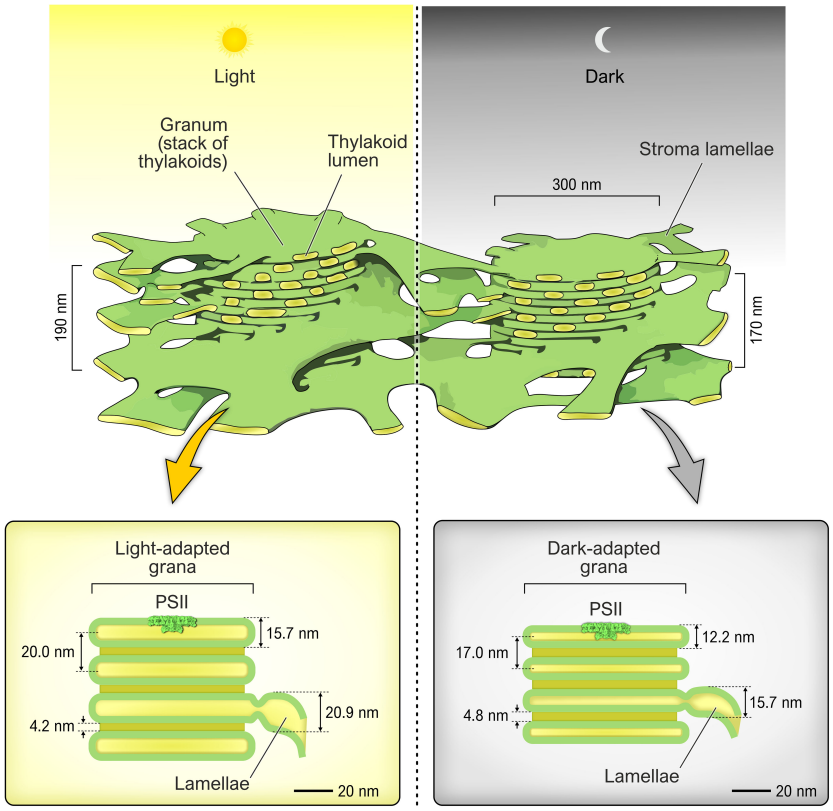

FIGURE 1
The top part shows two typical grana stacks in light (left) and dark (right) and their average sizes (modified from Staehelin and Paolillo, 2020). The lower part shows the distances of the various thylakoid membrane parts as Li et al. (2020) measured. The colour of the thylakoid lumen is yellow while the thylakoid membrane is green. The Photosystem II complex is shown in scale to the thylakoid membrane.

reassembled in the light? There have been reports that the lumen contains a pool of free extrinsic proteins (Hashimoto et al., 1996). As discussed in Kirchhoff et al., 2011, the PSII complexes cannot be in a face-to-face orientation, but instead they must be staggered. The shrinking of the lumen space in the dark will severely obstruct the movement of PSII, and presumably the movement of lumen proteins. This has been shown be the case for the electron linker between PSII and PSI, plastocyanin (Kirchhoff et al., 2011). If this could lead to that the "compression" of the lumen compartment reduce or even stop electron transport and or enzymatic activity in the dark, needs to be further investigated.

3 The lumen proteins

The thylakoid lumen is a highly unusual compartment with a proteome that is unique not only in the plant kingdom but also in the other kingdoms of life. Below is a summary of the different groups of proteins found in the lumen and Table 1 gives a summary.

3.1 Photosystem II associated proteins

At the lumenal side of PSII, three PSII subunits build the so-called Oxygen Evolving Complex (OEC) which, through its manganese cluster (Mn_4O_5Ca), catalyzes the oxidation of water. These extrinsic subunits, namely PsbO, PsbP, and PsbQ (Kieselbach et al., 1998a), each have specific roles in maintaining the functionality of PSII. Positioned in close proximity to the manganese cluster, the PsbO subunit (of which two forms O1 and O2 have been identified in *Arabidopsis*) is suggested to serve as a GTPase (regulating PSII repair) (Lundin et al., 2008; Spetea, 2012). Both PsbP and PsbQ (are present in several forms *vide infra*) play a general role in ensuring PSII stability, and they may cooperate in orchestrating the insertion/removal of the manganese cluster during the PSII repair cycle. For more details on these intensely studied extrinsic proteins see for instance reviews De Las Rivas et al. (2007); Bricker et al. (2012), and Roose et al. (2016).

Finally, there is the HCF136 protein, which is a nuclear-encoded protein regulating the mechanism underlying the biogenesis of PSII (Meurer et al., 1998; Yu et al., 2018).

3.2 PsbP-like proteins

As above-mentioned, the PsbP protein is an extrinsic component of Photosystem II (PSII), located on the lumenal side of the thylakoid membrane and involved in the lumenal part of the oxygen evolving complex in higher plants. In addition to these well-

studied extrinsic subunits of PSII, the thylakoid lumen contains a new protein family of PsbP-like proteins.

The PsbP gene family has ten members in the genome of the model plant *Arabidopsis thaliana*, with two encoding for true PsbP proteins (PsbP1 and PsbP2), and eight encoding PsbP-like proteins. Two of the eight homologs with the highest sequence similarity to PsbP1 have been classified as PsbP-like proteins (PPL1 and PPL2) (Liu et al., 2012), while the remaining six are referred to as PsbP-domain proteins (PPD). For the first two a distinct function has been assigned. In fact, the PPL1 is required for the efficient repair of photodamaged PSII and PPL2 is crucial for the accumulation of the chloroplastic NDH complex (Ishihara et al., 2007). On the contrary, no function has yet been found for any of the six PPD proteins but results from non-reducing/reducing electrophoresis indicate that at least some of these proteins might form complexes with other lumenal proteins. In addition, crystal structure studies suggests that the PsbP proteins bind metals (Cao et al., 2015).

3.3 PsbQ-like proteins

As in the previously mentioned scenario, there exists a group of PsbQ-like proteins that has undergone extensive investigation in *A. thaliana*. The NAD(P)H dehydrogenase-like (NDH) complex, which is involved in the PSI cyclic electron flow, has three lumenal PsbQ-like protein subunits referred to as PQL1, PQL2, and PQL3. While PQL3 is an assembly factor of the NDH, the other two proteins were shown to be essential for the functionality of NDH and tightly associate with the NDH-PSI supercomplex (Yabuta et al., 2010; Ishikawa et al., 2020). Nevertheless, there remains a lack of information regarding PsbQ-like proteins.

3.4 Cytochrome b_6f, plastocyanin, and PSI proteins

The electron transport activity is intricately linked to the pH of the lumen through a process known as the "Q-cycle" (Crofts, 2004). During this cycle, two protons are actively transported from the stromal side to the lumen. This process involves the utilization of a portion of the electrons produced by PSII in a series of consecutive reduction and oxidation reactions involving the plastoquinone (PQ), a membrane-soluble carrier responsible for transporting both electrons and protons. Subsequently, these electrons are transferred to the cytochrome b_6f complex (cyt b_6f), which features a specific subunit known as the Rieske protein. Although this is an integral thylakoid membrane protein, it possesses a binding domain for a lumen iron-sulphur cluster (2Fe-2S), eventually playing a pivotal role in facilitating the transfer of electrons between cytochrome b_6 and the cytochrome f within the complex (Carrell et al., 1997). The membrane located cyt b_6f complex then channels the electrons to plastocyanin (PC), a lumenal electron carrier containing copper. The PC acts as a conduit for the electrons, ultimately delivering them to the PSI. Two homologous PC (PLAS1 and PLAS2) isoforms have been identified in the lumen compartment of which PLAS2 is expressed at considerably higher level than PLAS1. However, knockout and over-expression mutants did not reveal any specific functional difference between the two forms (Pesaresi et al., 2009). The interaction between PC and PSI is mediated by the only identified lumenal subunit of PSI, referred to as PsaN, which is essential for ensuring an efficient electron flow. Eventually, the lumen localized cytochrome c6 like protein (AT5g45040) has been hypothesized to be an alternative electron carrier in the thylakoid lumen of *Arabidopsis* (Gupta et al., 2002), however this role is still controversial (Weigel et al., 2003).

3.5 Proteases

The thylakoid membrane hosts the four major complexes of the photosynthetic machinery, as most of their subunits are integral membrane proteins. To adapt to changing environmental conditions in which photosynthesis operates, the turnover of several subunits becomes essential to sustain this process. Within the chloroplast, several members of the Deg proteases, ATP-independent serine-type proteases diffused across the kingdoms of life, play a crucial role. There are two members in the stroma compartment (Deg2 and Deg7) and three in the lumen (Deg1, Deg5 and Deg8), each responsible for different degradation events (Schuhmann and Adamska, 2012; Knopf and Adam, 2018).

One significant event in this context is the turnover of the D1 subunit of PSII (Knopf and Adam, 2018). This process involves a two-step procedure: first, the degradation of the exhausted D1 subunit, followed by the incorporation of a newly synthesized one, leading to the reactivation of PSII. In the initial step, Deg1 plays an essential role as it cleaves the hydrophilic lumen-located segment of D1. Importantly, the activity of Deg1 is regulated by both pH and light. Deg1 forms an active homo-hexameric complex only under illumination when the lumen pH becomes acidic and via a dephosphorylation event, ensuring activation only in the presence of photodamage, specifically when D1 is damaged. Indeed, it is worth noting that Deg1 is not limited to the D1 subunit; it also participates in the degradation of other important photosynthetic proteins such as plastocyanin and PsbO, actively contributing to the maintenance of lumenal homeostasis.

On the other hand, the other two proteases, Deg 5 and Deg8, were observed to form a hetero-oligomeric complex with a 1:1 stoichiometry. These proteases are known to collaborate with Deg1 in the initial cleavage of D1 and may potentially play a role in the degradation of the PsbF subunit of PSII (Knopf and Adam, 2018).

3.6 Immunophilins

The immunophilin superfamily comprises a group of conserved and widely distributed proteins, consisting of two structurally distinct subfamilies, each sharing a common enzymatic activity: the cyclophilins and the FK506/rapamycin-binding proteins (FKBPs) (Gollan et al., 2012). Within land plants, immunophilins are found in various tissues and cellular compartments, including the chloroplast, where they are present in both the stroma and the

lumen (Buchanan and Luan, 2005). Several examples of these proteins include:

i) TLP40, initially isolated in spinach, serves as a folding catalyst and regulator of dephosphorylation in the turnover of the D1 subunit (Fulgosi et al., 1998);

ii) Cyclophilin 28 (CYP28), which plays a role in regulating the assembly and accumulation of PSII-LHCII supercomplexes by modifying the conformation of Lhcb6 through its peptidyl prolyl cis/trans isomerase (PPIase) activity (Zhu et al., 2022);

iii) Cyclophilin 38 (CYP38), which is critically involved in the assembly and maintenance of PSII supercomplexes. CYP38 also facilitates the proper folding of the D1 and CP43 (PsbC) subunits of PSII, contributing to the successful assembly of the Oxygen Evolving Complex (OEC) (Vasudevan et al., 2012). Therefore, immunophilins play vital roles in the functioning and structural maintenance of the photosynthetic machinery within chloroplasts.

3.7 Stress-related proteins

The thylakoid lumen also contains several proteins designed to address harmful conditions, such as the presence of Reactive Oxygen Species (ROS) (Petersson et al., 2006; Foyer, 2018). One notable example is the xanthophyll cycle, during which the conversion of violaxanthin to zeaxanthin is catalyzed by the lumenal enzyme violaxanthin de-epoxidase (VDE), utilizing ascorbate as a reducing agent (Simionato et al., 2015). As previously mentioned, when exposed to intense light, the pH of the lumen decreases. This change causes VDE activation through a transition from a soluble state to an association with the thylakoid membranes, where its substrate, violaxanthin, is located. Conversely, on the stromal side, zeaxanthin epoxidase (ZE) is responsible for the conversion back to violaxanthin when light intensity decreases. Another set of pH-sensing events is associated with the PsbS protein, a 22kDa membrane protein with a multifaceted activity related mainly to PSII protection (Nicol and Croce, 2021; Fantuzzi et al., 2023). While it is not a lumen protein *per se*, it possesses a domain whose functionality is linked to the lumen side of the thylakoid membranes.

3.8 Pentapeptide repeat proteins

The pentapeptide repeat domain consists of tandem repeats of five amino acids, with each such repeat forming a beta-strand and constituting a quadrangle. Most PRPs are found in prokaryotes. The genome of *Arabidopsis thaliana* encodes for six pentapeptide repeat proteins, of which three are located in the thylakoid lumen: TL15, TL17 and TL20.3 (Kieselbach et al., 1998b). Interestingly, these PRP proteins stand out by having more than 50% predicted intrinsic disorder but no clear function has been assigned yet.

3.9 Proteins with unknown function

Surprisingly, after 25 years of research, no function has been assigned to the largest protein group in the lumen (at least 28 proteins). This show that the bottle neck in biochemistry of proteins is to find functions for them. Traditionally, to understand the function of a protein, knock-out mutants are created and the mutant plant is subjected to various stresses to determine whether a particular phenotype could be associated with the specific mutation. However, for many proteins located in the chloroplast lumen, a knock-out mutant often does not show a clear phenotype. Instead, when a phenotype is observed, questions about secondary effects are raised. One exception from this is the hcf136 mutant in which the amount of PSII is strongly reduced (Plücken et al., 2002). On the other end is the case of the TL29 protein. It is one of the most abundant proteins in the thylakoid lumen, has sequence and structural similarities typical to ascorbate peroxidases, and due to this it was actually renamed by some researchers to APX4. The TL29 protein is expressed at higher levels in light and seems to be mainly in the grana lumen close to PSII. However, crystallisation of the protein revealed that TL29 has an additional loop in the suggested active site that make the binding of the catalytically heme group impossible (Granlund et al., 2009). The isolated TL29 protein does not bind any heme group, it cannot be reconstituted with a heme group and does not have any peroxidase activity (Granlund et al., 2009). In summary we know many features of the TL29 protein, except its function.

We are entering a new era where not only protein sequences but also structure predictions and searches can be made by Artificial intelligence (AI). The AlphaFold database (Varadi et al., 2022) that contains the dizzying amount of 214 million predicted proteins can be searched using programs such as Foldseek (Barrio-Hernandez et al., 2023). Obtaining or prediction of the structures for the proteins in the lumen with unknown function could through this type of searches identify remote structural similarity and identify domain families. This could then lead to clues for their function and an opening to better understand the thylakoid lumen proteome.

4 The function of lumen compartment

There is clear evidence that the chloroplast lumen is closely linked to plant cell signalling. For instance, most lumenal proteins are light-dependently co-expressed (Granlund et al., 2009) and respond to cold (Goulas et al., 2006). The extrinsic PsbP protein is considerably affected by viral infection and oxidative stress (Pérez-Bueno et al., 2004; Karamoko et al., 2011). In addition, there is evidence that the function of the STN7 kinase depends on the correct folding of its C-terminus through its lumenal disulfide bridge (Lemeille et al., 2009; Kieselbach, personal observation). There is also clear evidence that lumenal proteins have important functions in the assembly of photosystem II (Meurer et al., 1998; Plücken et al., 2002; Che et al., 2013) and its supercomplexes (Lima et al., 2006; Fu et al., 2007), but little is known about the details and the time course of the assembly process. For instance, the lumenal chloroplast proteome of *Arabidopsis*

thaliana has eight immunophilins, which is the highest number known for a subcellular compartment. In addition, it also includes ten PsbP proteins that are unique to the thylakoid lumen and three pentapeptide repeat proteins, accounting for half of the members of this protein family in *Arabidopsis*. Furthermore, 20 of the 45 experimentally confirmed lumenal proteins of *Arabidopsis* (which is more than 40%) are *in vitro* targets of thioredoxin (Hall et al., 2010), which are enzymes found in each compartment of the chloroplast except for the inner space for the envelope (Kang and Wang, 2016). For a comparison, in the chloroplast stroma, about 2% of the known proteins are *in vitro* targets of thioredoxin (Lindahl and Kieselbach, 2009) even though the stroma contains an extended family of thioredoxins (Cain et al., 2009). This suggests that the lumen is important for thioredoxin-dependent signalling pathways. In summary, the unique features of the lumen chloroplast compartment suggest that it is a rich source for the discovery of novel knowledge and regulatory interactions between proteins.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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