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ARTICLE *in* FEBS LETTERS · JUNE 2003

Impact Factor: 3.17 · DOI: 10.1016/S0014-5793(03)00366-1 · Source: PubMed

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The major peptidyl-prolyl isomerase activity in thylakoid lumen of plant chloroplasts belongs to a novel cyclophilin TLP20

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Received 28 February 2003; accepted 27 March 2003

First published online 15 April 2003

Edited by Richard Cogdell

Abstract Fractionation of proteins from the thylakoid lumen of spinach chloroplasts combined with peptidyl-prolyl *cis/trans* isomerase (PPIase) measurements revealed a major isomerase activity that was ascribed to a novel enzyme TLP20 (thylakoid lumen PPIase of 20 kDa). TLP20 was inhibited by cyclosporin A and mass spectrometric sequencing of tryptic peptides confirmed its classification as a cyclophilin. Genes encoding similar putative thylakoid cyclophilins with a unique insert of three amino acids NPV in their N-termini were found in chromosome 5 of both *Arabidopsis* and rice. TLP20 is suggested to be the major PPIase and protein folding catalyst in the thylakoid lumen of plant chloroplasts.

Key words: Cyclophilin; Mass spectrometry; Peptidyl-prolyl isomerase activity; Spinach; Thylakoid lumen

1. Introduction

The light-driven reactions of plant photosynthesis take place in the thylakoid membrane of chloroplasts, which is the best functionally and structurally studied membrane protein system from a bioenergetic point of view [1,2]. The thylakoids form a continuous membrane network enclosing an inner space designated the thylakoid lumen [3]. This cellular compartment has become increasingly interesting with the advances in understanding regulation and stress protection of the photosynthetic process. Until recently the thylakoid lumen was considered to contain just a few soluble proteins facilitating electron transfer and oxidation of water, but recent proteomic studies have revealed at least 80 different proteins to be present in the lumenal space [3–6]. Unexpectedly, the largest group of these proteins was classified as peptidyl-prolyl *cis/trans* isomerases (PPIases) [5,6].

PPIases catalyze isomerization around peptidyl-prolyl imide bonds in peptides and proteins serving as protein folding catalysts [7,8] as well as regulators of diverse cellular processes including cell signaling, biogenesis and activities of several receptors (reviewed in [9,10]). These enzymes belong to three families: cyclophilins, FK506 binding proteins (FKBPs) and parvulins [9]. The cyclophilins and FKBPs are also collectively called immunophilins because they bind the immunosuppressive drugs cyclosporin A (CsA) and FK506, respectively, which inhibit their PPIase activity [11]. The catalytic domains of PPIases have a size between 10 and 20 kDa. There are also complex PPIases typically sized over 35 kDa with several binding modules linked to the PPIase catalytic domains [9], which determine substrate-dependent activity and specialized functions for these complex PPIases [12–14].

The proteomic study by Schubert and colleagues [6] revealed three putative cyclophilins and five FKBPs in the thylakoid lumen from *Arabidopsis thaliana*. The genome-based analysis by the same authors predicted as many as 10 PPIases in the lumen [6]. The study by Peltier and colleagues [5] identified five and predicted at least four additional putative PPIases in the same compartment. Thus, involvement of several PPIases in protein folding was proposed as one of the prime functions of the lumenal proteome [5,9]. However, the only active PPIase from the thylakoid lumen experimentally characterized prior to the present study is a cyclophilin-like protein TLP40 (thylakoid lumen PPIase of 40 kDa) [15]. This complex cyclophilin has a C-terminal PPIase catalytic domain and an N-terminal domain with a number of protein binding modules [9,15–17]. TLP40 has been shown to interact with the thylakoid membrane and regulate the activity of the photosystem II-specific protein phosphatase [15,17,18].

In the present work we used a prolyl-containing peptide for the assay of PPIase activity to address the enzymatic issue of the multiple PPIases in the thylakoid lumen. We demonstrate that the major PPIase activity from the lumen of spinach thylakoids belongs to a novel cyclophilin designated TLP20 (thylakoid lumen PPIase of 20 kDa). We also find the genes for cognate cyclophilins in the genomes of *Arabidopsis thaliana* and *Oryza sativa*. Our data suggest that TLP20 may be a general protein folding catalyst, while the other putative PPIases in the thylakoid lumen could serve more specialized regulatory functions as has been implied for the complex cyclophilin TLP40.

2. Materials and methods

2.1. Plant material and isolation of thylakoid lumen

Spinach (*Spinacea oleracea*) was grown hydroponically at 20–25°C at a light intensity of 400–500 μmol photons/m^2/s with a 10/14 h light/dark regime. Soluble lumenal proteins were purified according to Kieselbach et al. [3]. The buffer used during the Yeda press treatment was changed to 20 mM Tricine pH 7.8, 5 mM $MgCl_2$, 100 mM sucrose for fractionation of PPIases and to 50 mM 2-(*N*-morpholino)ethanesul-

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Abbreviations: CsA, cyclosporin A; FKBP, FK506 binding protein; PPIase, peptidyl-prolyl *cis/trans* isomerase; TLP20, thylakoid lumen PPIase of 20 kDa; TLP40, thylakoid lumen PPIase of 40 kDa

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00366-1

fonic acid pH 6.0, 50 mM NaCl, 100 mM sucrose for TLP20 purification.

2.2. Fractionation of PPIases from the lumen and purification of TLP20

For a general fractionation of PPIases the lumenal proteins were separated on a Resource Q column (Amersham Biosciences) equilibrated with 20 mM Tricine pH 7.8 and eluted with a 0–1 M NaCl gradient. For purification of TLP20 the lumenal proteins were first applied on a Sepharose Q column (Amersham Biosciences) equilibrated with 50 mM 2-(*N*-morpholino)ethanesulfonic acid pH 6.0. TLP20 was eluted with unbound proteins while the other PPIases were left bound to the column. The TLP20-containing fraction was then applied on a Poros HS column (Applied Biosystems) equilibrated with the same buffer and subjected to fractionation upon application of a 0–1 M NaCl gradient.

2.3. PPIase assay

The PPIase activity was measured in a coupled reaction with chymotrypsin as described by Fischer et al. [19]. The peptide substrate *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide (Calbiochem) was dissolved in trifluoroethanol with the addition of LiCl to increase the proportion of *cis* isomer [20]. The reactions were started by peptide substrate addition to a mixture of protein sample and chymotrypsin in 0.1 M Tricine pH 7.8. The change in absorbance at 390 nm due to the release of *p*-nitroanilide was monitored using a Shimadzu UV-3000 spectrophotometer. In order to assay the activity in the presence of CsA or FK506 the protein sample was incubated with inhibitor for approximately 2 min before the reaction was started.

2.4. Protein separation, digestion with trypsin and mass spectrometric analyses

The fractions after chromatography were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis [21] using 15% acrylamide gels. For in-gel digestion the protein bands were excised from the gel and treated with trypsin (Sequencing Grade Modified, Promega) essentially according to the procedure described by Shevchenko et al. [22]. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analyses were carried out on Voyager-DE Pro (Applied Biosystems, Framingham, MA, USA) with recording of the reflector mass spectra and external calibration. Electrospray ionization tandem mass spectra were acquired on a hybrid mass spectrometer API Q-STAR Pulsar i (Applied Biosystems, Foster City, CA, USA) equipped with a nanoelectrospray ion source (MDS Protana, Odense, Denmark). The nanoelectrospray capillaries were loaded with 2 μl peptide solution in water/acetonitrile (50/50) with 1% formic acid. The collision-induced decomposition of selected peptide ions was performed using the instrument settings recommended by Applied Biosystems.

3. Results

To analyze the PPIase activity in the thylakoid lumen we used a conventional assay with the peptide substrate *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide [20]. The lumenal proteins were obtained from spinach thylakoids by the method of Kieselbach et al. [3], which involves extensive washing of the thylakoids before disruption of the membranes by Yeda press. No PPIase activity was detected in the washed thylakoids before membrane disruption and the activity appeared only when soluble proteins were released from the lumen. To make a general fractionation of the PPIase activities the lumenal proteins were subjected to anion-exchange chromatography on a Resource Q column, which led to a wide separation of proteins and PPIase activities along the salt gradient (Fig. 1). No PPIase activity was detected in the void with unbound proteins, while the most significant and distinct peak of the activity was eluted from the column at a low ionic strength (Fig. 1). This first PPIase peak reproducibly corresponded to approximately half of the total PPIase activity. The rest of the

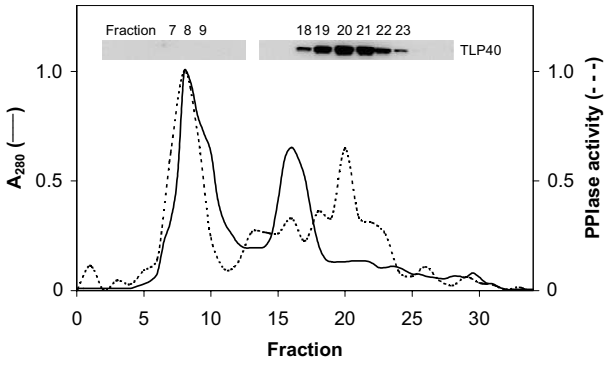

Fig. 1. Fractionation of PPIase activities from spinach thylakoid lumen. The proteins (continuous line) and PPIase activity (dashed line) from the thylakoid lumen were separated by anion-exchange chromatography on a Resource Q column. The inset shows the content of TLP40 in different fractions as determined by Western blotting analysis.

activity was distributed between the lower peaks along the broad range of the following fractions (Fig. 1). For further analysis the fractions were subjected to immunoblotting using a TLP40-specific antibody (Fig. 1, inset), since TLP40 was the only active PPIase from thylakoid lumen characterized before the present study [15,17]. Notably, TLP40 was found not in the fractions containing the first most active PPIase peak but in the later eluted peak containing a lower activity (Fig. 1). Thus, we concluded that the major PPIase activity of the thylakoid lumen belonged to a yet unknown PPIase distinct from TLP40.

To enrich and characterize the PPIase of the major peak we exploited its relatively weak retention during the anion-exchange chromatography (Fig. 1). To this end the lumenal proteins were applied onto the anion-exchange column in a slightly acidic conditions and the enzyme of interest was eluted in the void with unbound proteins, while the other PPIases including TLP40 were retained on the column (data not shown). The fraction containing released PPIase activity was subjected to cation-exchange chromatography, which yielded a single peak of PPIase activity (Fig. 2A). The most active fraction 10 contained only four proteins shown in the inset of Fig. 2A. The size of the proteins in this fraction was in the range between 20 kDa and 26 kDa. We quantified the relative amount of each of these four proteins in the fractions covering the peak of the PPIase activity. Fig. 2B demonstrates that the PPIase activity closely follows the protein with the molecular mass of 20 kDa. The sequencing of this 20 kDa protein confirmed that it is a PPIase (see below). Accordingly, we named this protein TLP20.

To determine the nature of TLP20 we tested its PPIase activity for inhibition by the immunosuppressive drugs CsA and FK506, which are inhibitors of cyclophilins and FKBPs, respectively [11]. Fig. 2C demonstrates that the activity of TLP20 was inhibited at low concentrations (nM) of CsA, however, no inhibition of the activity by FK506 was detected even at μM concentrations. These results demonstrate that TLP20 belongs to the cyclophilin family of PPIases and is substantially more susceptible to inhibition by CsA than TLP40, which is insensitive to CsA up to 10 μM [15,17].

To obtain more information about TLP20, the 20 kDa polypeptide band (Fig. 2A, insert) was excised from the gel and subjected to in-gel digestion with trypsin for further char-

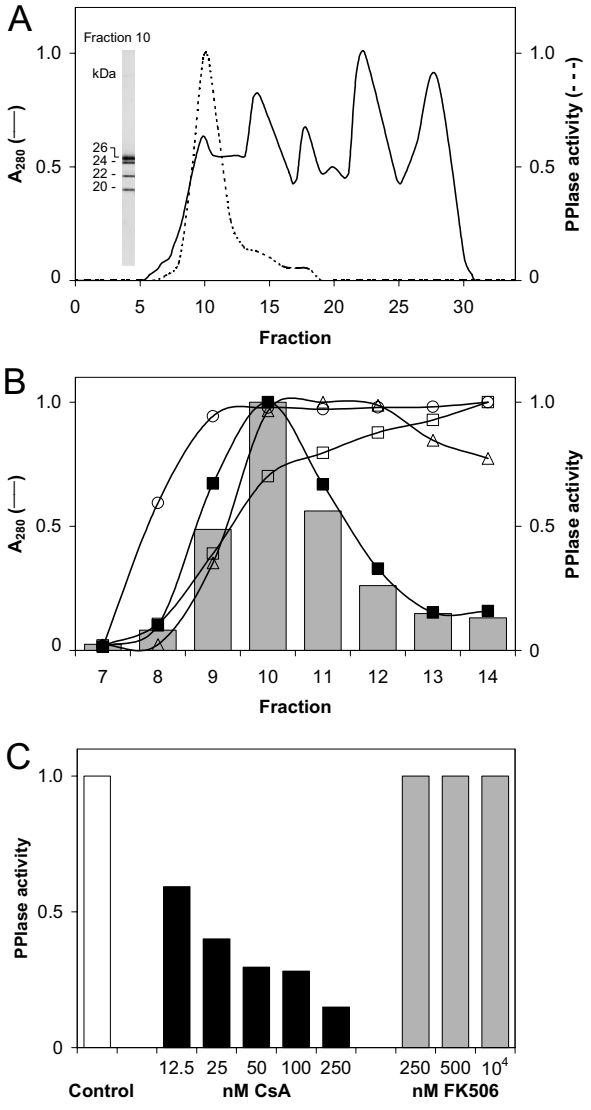

Fig. 2. Identification of the major PPIase from the thylakoid lumen as a cyclophilin (TLP20). A: Cation-exchange chromatographic separation of the fraction containing the major PPIase activity after anion-exchange chromatography. The protein elution profile is shown by a continuous line and the PPIase activity by a dashed line. The inset shows a silver-stained gel with proteins in the fraction 10. B: Correlation between the PPIase activity (gray bars) and the relative protein abundance (■, 20 kDa protein; △, 22 kDa protein; □, 24 kDa protein; ○, 26 kDa protein) in the fractions after the cation-exchange chromatography. C: Dependence of the PPIase activity in fraction 10 on the addition of increasing concentrations of cyclophilin inhibitor CsA and FKBP inhibitor FK506.

acterization by mass spectrometry. The peptides were initially analyzed by MALDI-TOF mass spectrometry, but the obtained peptide mass fingerprint did not match any known protein in the databases. This demonstrated that the sequence of this lumenal cyclophilin from spinach was not known previously and de novo sequencing of the TLP20 peptide fragments was required for identification of homologous proteins from the available genomic databases (*A. thaliana* and *O. sativa*) using amino acid sequence search. Thus, the peptides obtained from the tryptic digest of TLP20 were analyzed by electrospray ionization mass spectrometry. Three peptides were selected and subjected to collision-induced fragmentation (Fig. 3). The molecular ions of these peptides produced clear patterns of C-terminal and N-terminal fragment ions that allowed complete de novo sequencing. Fig. 3 shows the fragmentation spectra with the corresponding sequences for 10, 13 and 18 amino acid peptides from TLP20. A BLAST search [23] revealed that the first peptide (Fig. 3A) corresponded to the 20 amino acid peptide (SwissProt accession number P82536) obtained earlier by N-terminal sequencing of the 'thylakoid lumenal 18 kDa protein' from spinach [3]. This can be considered independent confirmation of the localization of TLP20 in the thylakoid lumen. The other two peptides were highly homologous to the conserved regions of numerous cyclophilins (Fig. 4 and data not shown). Thus, along with the inhibition experiments presented above, the sequencing information corroborated that TLP20 is a cyclophilin.

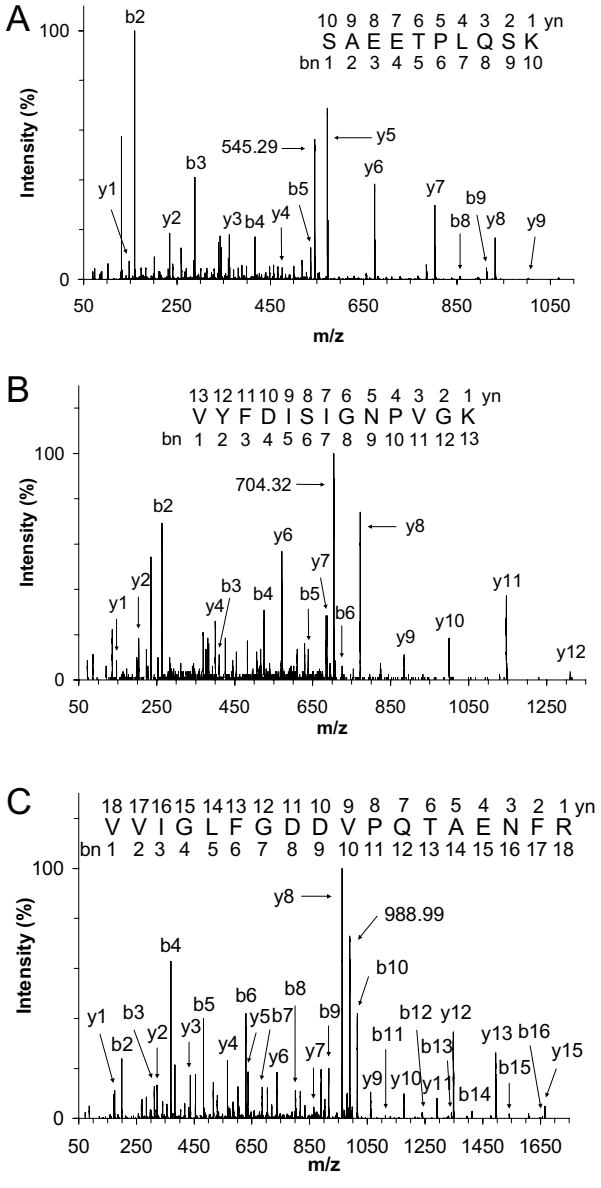

Fig. 3. Sequencing of tryptic peptides from TLP20 by electrospray ionization and collision-induced dissociation of their molecular ions. The fragmentation spectra of doubly charged peptides $(M+2H)^{2+}$ with *m/z* 545.29 (A), *m/z* 704.32 (B) and *m/z* 988.99 (C). The major fragment b-ions (N-terminal) and y-ions (C-terminal) are indicated in each spectrum together with the peptide amino acid sequences revealed after the fragmentation.

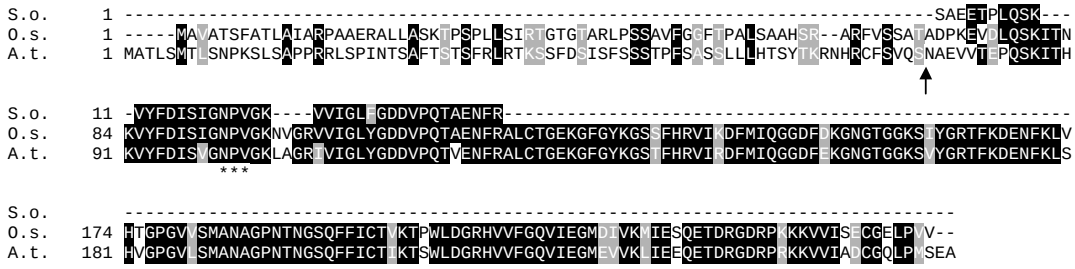

Fig. 4. Sequence alignment of three peptides from spinach TLP20, *S. oleracea* (S.o.), with homologous putative proteins from *A. thaliana* (A.t.) (TIGR At5g13120) and *O. sativa* (O.s.) (TIGR gene temp_id 2972.t00007). Black and gray boxes represent identical and similar amino acids, respectively. The cleavage site of the signalling peptide for the *Arabidopsis* precursor protein is indicated by the arrow. The amino acid insert NPV unique for these three cyclophilins is indicated by asterisks.

The database sequence search with all three TLP20 peptides (in total 41 amino acid residues) allowed identification of cognate putative precursor proteins encoded in the genomes of *Arabidopsis* and rice. These genes are encoded in chromosome 5 in both *Arabidopsis* (TIGR Gene locus At5g13120) and rice (TIGR Gene temp_id 2972.t00007). The alignment of these two putative protein sequences with the sequenced peptides from TLP20 is shown in Fig. 4. Both ChloroP [24] and TargetP [25] algorithms predicted the chloroplast as the site of subcellular localization for the putative TLP20 from both *Arabidopsis* and rice. The ChloroP algorithm predicted the signal peptide cleavage site at amino acids 56–57 and 70–71 for the precursor proteins from rice and *Arabidopsis*, respectively. However, the recent N-terminal sequencing of the *Arabidopsis* protein found in the thylakoid lumen [6] revealed the real cleavage site between amino acids 75 and 76, as indicated by the arrow in Fig. 4.

Notably, the sequences of the TLP20 proteins from spinach, *Arabidopsis* and rice contain a unique three amino acid insert NPV in the N-terminal region (indicated with asterisks in Fig. 4), which makes them distinct from all other known cyclophilins. It is highly possible that the distinctive NPV insert (Fig. 4) could be a fingerprint motif for TLP20-like cyclophilins from thylakoid lumen of different plant species.

4. Discussion

In this study we have focused on the characterization of the major PPIase activity in the chloroplast thylakoid lumen. The first enzyme with PPIase activity isolated from the thylakoid lumen was a complex cyclophilin-like protein, TLP40, which has been proposed to be responsible for PPIase activity and protein folding in the lumen in addition to its regulatory function [15,17]. However, our present study demonstrates that TLP40 does not determine the major PPIase activity in this cellular compartment. Instead, we show that a novel PPIase, TLP20, is responsible for the main prolyl isomerase activity in the thylakoid lumen. TLP20 belongs to the cyclophilin family of PPIases: its enzymatic activity is inhibited by nanomolar concentrations of CsA and the protein has a high sequence identity to other cyclophilins. TLP20 has a molecular mass of 20 kDa, which is the conventional size for cyclophilins containing a single PPIase domain [9]. In contrast, TLP40 is a complex PPIase with a cyclophilin-like domain and additional binding modules responsible for its interactions with the thylakoid membrane proteins [9,15,17]. Moreover, TLP40 associates and co-purifies with the thylakoid membrane protein phosphatase [15,17] and the reversible interaction of TLP40 with the protein phosphatase regulates the rate of dephosphorylation for thylakoid membrane phosphoproteins [17]. This interaction has also been implied in the fast dephosphorylation of photosystem II reaction center proteins in heat shock conditions [18]. Thus, TLP40 has a specialized regulatory function(s), while TLP20 may be the general protein folding catalyst in the lumen of chloroplast thylakoids.

We identified the genes encoding putative TLP20-like precursor proteins in chromosome 5 of both *Arabidopsis* and rice. These precursor proteins contain chloroplast targeting signal peptides and the mature homolog of TLP20 has recently been found among the proteins of the thylakoid lumen of *Arabidopsis* by proteomic analysis [6]. Our finding of the unique three amino acid insert NPV in the amino-termini of the TLP20 proteins from spinach, rice and *Arabidopsis* provides a strong argument for the similarity of these particular lumenal cyclophilins. The NPV insert makes these three PPIases distinct from all other known cyclophilins. This unique sequence stretch may be a specific marker for identification of TLP20-like lumenal cyclophilins in other plant species.

The proteomic analyses identified or predicted 13 PPIases in the lumen: four cyclophilins and nine FKBPs [6]. It is a very great number taking into account the relatively tiny volume occupied by this cellular compartment and that only 22 FKBPs are encoded in the whole *Arabidopsis* genome [6]. The identification of TLP20 as the most active lumenal PPIase suggests that the other PPIases could have rather specialized functions and/or restricted substrate specificity, but not just contribute to the sum of the strong protein (un)folding activity inside thylakoids as has been recently proposed [5]. At present, we suggest that TLP20 is responsible for the common protein folding catalysis, while TLP40 and the other putative PPIases could have rather restricted substrate specificity and dedicated binding partners. The yet uncharacterized high number of putative PPIases in the thylakoid lumen likely serve specific regulatory functions and elucidation of these functions may contribute to the rapidly increasing appreciation of the significance of the thylakoid lumen in the maintenance and regulation of the photosynthetic process.

Acknowledgements: This work was supported by grants from The Swedish Research Council, The Swedish Research Council for Environment, Agriculture and Spatial Planning (Formas) and Nordiskt Kontaktorgan för Jordbruksforskning (NKJ).

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