The EMBO Journal Vol.17 No.6 pp.1577–1587, 1998

A novel multi-functional chloroplast protein: identification of a 40 kDa immunophilin-like protein located in the thylakoid lumen

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We describe the identification of the first immunophilin associated with the photosynthetic membrane of chloroplasts. This complex 40 kDa immunophilin, designated TLP40 (thylakoid lumen PPIase), located in the lumen of the thylakoids, was found to play a dual role in photosynthesis involving both biogenesis and intra-organelle signalling. It originates in a single-copy nuclear gene, is made as a precursor of 49.2 kDa with a bipartite lumenal targeting transit peptide, and is characterized by a structure including a cyclophilin-like C-terminal segment of 20 kDa, a predicted N-terminal leucine zipper and a potential phosphatase-binding domain. It can exist in different oligomeric conformations and attach to the inner membrane surface. It is confined predominantly to the non-appressed thylakoid regions, the site of protein integration into the photosynthetic membrane. The isolated protein possesses peptidyl-prolyl *cis–trans* isomerase protein folding activity characteristic of immunophilins, but is not inhibited by cyclosporin A. TLP40 also exerts an effect on dephosphorylation of several key proteins of photosystem II, probably as a constituent of a transmembrane signal transduction chain. This first evidence for a direct role of immunophilins in a photoautotrophic process suggests that light-mediated protein phosphorylation in photosynthetic membranes and the role of the thylakoid lumen are substantially more complex than anticipated.

Keywords: cyclophilin/PPIase/protein folding/thylakoid lumen/thylakoid protein phosphatase

Introduction

Thylakoid membranes in chloroplasts catalyse the fundamental process of photosynthetic energy conversion. Outstanding characteristics of this specialized biomembrane are its dual genetic origin (Herrmann, 1996) and the enormous physiological versatility, in particular its ability to manage short- and long-term changes in the light environment (Andersson and Barber, 1994). Various mechanisms can regulate the distribution of excitation energy between the two photosystems, and others can convert excess excitation energy into thermal energy. Furthermore, a regulated protein turnover can re-establish function once photodamage has occurred, or acclimatize the photosynthetic machinery to seasonal changes. A multitude of auxiliary enzymes (Andersson, 1992) are involved in these physiological processes. These include, for instance, kinases and phosphatases that control the energy distribution between the two photosystems (Allen, 1992; Gal *et al.*, 1997), a substantial number of proteases that catalyse regulated protein degradation (Adam, 1996; Sokolenko *et al.*, 1997) and various protein components that are required during biogenesis of the photosynthetic multisubunit complexes (Herrmann, 1996; Robinson and Knott, 1996). The identification of such auxiliary components and their genes, and in particular the elucidation of the signal transducing mechanisms connecting light with physiological responses, are central and developing topics of current research in photosynthesis.

Reversible protein phosphorylation is a primary means of mediating signal transduction in living organisms. In chloroplast thylakoids, nearly a dozen polypeptides, mostly components of the photosystem II complex and its major chlorophyll *a*/*b*-binding protein LHCII, can be phosphorylated reversibly in a redox-controlled manner (Allen, 1992; Gal *et al.*, 1997; Vener *et al.*, 1997). Thylakoid protein phosphorylation ensures the energy balance between the two photosystems (Allen, 1992) and regulates protein turnover during the repair of photoinhibitory damage to photosystem II (Aro *et al.*, 1993; Andersson and Aro, 1997). Although the physiology of thylakoid protein phosphorylation is well characterized in many respects, surprisingly little is known about the nature of the components involved, their biogenesis, regulation and enzymology. In studying these aspects, we have been able to identify a novel auxiliary enzyme, a 40 kDa immunophilin-like protein associated with the thylakoid system and possessing several unique features.

Immunophilins comprise a superfamily of conserved ubiquitous proteins consisting of two distinct subfamilies; cyclophilins and FKBPs (FK506/rapamycin-binding proteins), the targets of the immunosuppressive drugs cyclosporin A (CsA) and FK506/rapamycin, respectively (see Schreiber, 1991). Despite the lack of structural similarity, all cyclophilins and FKBPs share a common enzymatic, so-called PPIase or rotamase activity, catalysing *cis–trans* isomerization of proline imidic peptide bonds (Fischer *et al.*, 1989). A more recently discovered third group of proteins (parvulins) which is insensitive to immunosuppressive drugs also possesses PPIase activity (Rahfeld *et al.*, 1994; Hani *et al.*, 1995; Rudd *et al.*, 1995). The molecular masses of cyclophilins, FKBPs and parvulins range normally in the order of 18–21 kDa, 12–13 kDa and 10–13 kDa respectively, but a few complex immuno-

philins of higher molecular weight have been detected recently (Hani *et al.*, 1995; Rudd *et al.*, 1995; Marks, 1996).

The biological significance of this group of enzymes is a matter of intense research. Acceleration of protein folding processes by PPIases *in vitro* (see Fruman *et al.*, 1994) and *in vivo* (Matouschek *et al.*, 1995; Rassow *et al.*, 1995) has supported a physiological role as folding catalysts facilitating the slow and generally rate-limiting uncatalysed isomerization around Xaa–Pro peptide bonds (Brandts *et al.*, 1975; Schmid and Baldwin, 1978; Lang *et al.*, 1987). Immunophilins can also perform chaperone functions (Freskgard *et al.*, 1992) or cooperate with other chaperone proteins.

However, PPIases are not only involved in the folding and assembly of newly synthesized or translocated proteins but exert a second major role in cellular signalling (Schreiber, 1992; Walsh *et al.*, 1992). In this latter aspect, they participate in the regulation of an array of quite diverse, crucial cellular functions, in addition to intracellular protein trafficking (Klappa *et al.*, 1995; Matouschek *et al.*, 1995; Rassow *et al.*, 1995), such as the control of mitosis (Lu *et al.*, 1996), RNA maturation (Hani *et al.*, 1995; Mi *et al.*, 1996), interaction with viral components (Luban *et al.*, 1993), DNA degradation during apoptosis (Montague *et al.*, 1997) or modulation of calcium release channels (see Marks, 1996). Complex immunophilins, such as the 40 kDa Cyp-40 cyclophilin and the 52 kDa FKBP52, may be constituents of supra-molecular structures, as has been shown for the human steroid receptor complex (Pratt, 1993) or chaperone supercomplexes (Bose *et al.*, 1996; Freeman *et al.*, 1996), and to be involved in Hsp90-dependent signal transduction (Duina *et al.*, 1996).

Various members of the immunophilins are involved in phosphorylation processes via transient interaction with kinases (Abraham, 1996) and phosphatases (see Schreiber, 1992). The details of these interactions are not yet understood, except for the case of calcineurin (Bierer *et al.*, 1990; Schreiber, 1992; Walsh *et al.*, 1992). In mammals, the Ca^{2+}-calmodulin-dependent heterodimeric protein phosphatase calcineurin can bind immunophilins with complexed immunosuppressive drugs. This interaction inhibits the protein phosphatase activity, resulting in interruption of the signal transduction cascades required for T-cell activation, which in turn suppresses the immunoresponse (see Schreiber, 1992). In yeast, immunophilins were found to interact with calcineurin in the absence of exogenous immunosuppressive ligands (Cardenas *et al.*, 1994).

In higher plants, immunophilins recently have been identified in various tissues and cellular compartments (Lippuner *et al.*, 1994; Luan *et al.*, 1994a,b, 1996; Sheldon and Venis, 1996). In chloroplasts, a 20 kDa cyclophilin and a 13 kDa FKBP were demonstrated to reside in the soluble stroma compartment (Lippuner *et al.*, 1994; Luan *et al.*, 1994a,b). In this study, we present molecular biological and biochemical data on a high molecular weight, 40 kDa, cyclophilin-like protein in plants and its association with the thylakoid membrane system. This complex immunophilin uniquely has its location in the thylakoid lumen and exhibits multifunctional properties with both peptidyl prolyl *cis–trans* isomerase protein folding activity and a regulatory effect on thylakoid protein dephosphorylation. This protein has been designated TLP40 (thylakoid lumen PPIase).

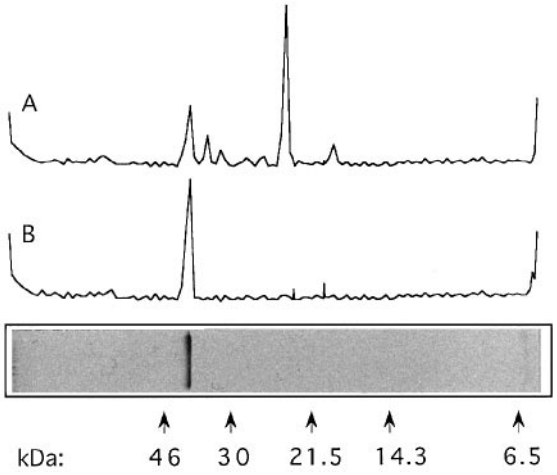

Fig. 1. Identification of a 40 kDa protein isolated from spinach thylakoids. Laser densitometric scans of a silver-stained SDS–polyacrylamide gel with: (**A**) a fraction after four steps of anion-exchange chromatography, containing the 40 kDa protein, several additional polypeptides and the phosphatase activity; (**B**) purified 40 kDa thylakoid protein after the final chromatographic step on Superose 12. The photograph of the corresponding silver-stained gel lane is shown below the scan. The positions of molecular mass markers are indicated.

Results

Isolation and microsequencing of a 40 kDa thylakoid protein

During the course of work aimed at purification and characterization of thylakoid protein phosphatases, we have identified and isolated a novel low abundance 40 kDa protein from spinach thylakoids. Extraction of isolated thylakoid membranes with Triton X-100 followed by anion-exchange chromatography with successive detergent replacements from Triton X-100 to *N*-dodecyl-β-D-maltoside and CHAPS yielded a >500-fold phosphatase-enriched fraction. This chromatographic fraction contained ~6 proteins including a rather prominent 40 kDa polypeptide as judged from SDS–PAGE (Figure 1A). A subsequent gel filtration step of this fraction resulted in further purification and yielded a preparation with pure 40 kDa protein (Figure 1B). Although this polypeptide in the final chromatographic step no longer eluted with the thylakoid protein phosphatase activity, its obstinate association with this enzyme during all the previous purification steps prompted further analysis.

To explore its nature and molecular properties, the 40 kDa polypeptide was subjected to microsequencing. An N-terminal and three internal peptide sequences resulting from proteolytic fragments generated after cyanogen bromide cleavage were obtained (Figure 2). Sequence comparisons gave no clear match apart from a restricted local similarity (30–60% identity) to a 'hypothetical protein', designated D64001, of the cyanobacterium *Synechocystis* sp. and an 'unknown protein', U59235, of another cyanobacterium, *Synechococcus* PCC7942.

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MSSFINHHFY PSVCTSKHAL PINPTSPFYL GIPNFRQKSR FMHLTPRCFS   50

RQIDPLDKQK KRSFSVKECA ISLALAAALI SGVPSLSWER HAEALTSPVL  100

PDLAVLISGP PIKDPEALLR YALPIDNKAI REVQKPLEDI TESLRVLGLK  150

ALDSVERNLK QASRALKNGK SLIIAGLAES KKDRGVELLD KLEAGMGELQ  200

QIVENRNREG VAPKQRELLQ YVGSVEEDMV DGFPYEVPEE YQTMPLLKGR  250

AVVEMKVKVK DNPNVDNCVF RIVLDGYNAP VTAGNFLDLV ERHFYDGMEI  300

QRRDGFVVQT GDPEGPAEGF IDPSTEKPRT IPLEIMVEGE KVPVYGSTLE  350

ELGLYKAQTK LPFNAFGTMA MAREEFENNS GSSQIFWLLK ESELTPSNAN  400

ILDGRYAVFG YVTDNQDYLA DLKVGDVIES VQAVSGVDNL VNPTYKIAG   449
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Fig. 2. Deduced amino acid sequence of the entire precursor TLP40 protein of spinach chloroplasts (DDBJ/EMBL/GenBank databank accession No. Y12071). The terminal processing site of the precursor is marked by an arrow. The N-terminal and three internal sequences, resulting from cyanogen bromide cleavage of purified TLP40, obtained by microsequencing are underlined. Seven-residue repeat regions of a putative leucine zipper are boxed. The two stretches of high similarity to the sequence GKKFDSSRDRN[43] in the immunophilin FKBP12, responsible for the binding to the protein phosphatase calcineurin (Aldape *et al.*, 1992), are highlighted.

Isolation and characterization of cDNA encoding a novel thylakoid precursor protein

Via PCR with degenerated primers designed against the microsequenced protein and peptides (using 17 bp terminal and 17 bp internal polypeptide sequences), a single 693 bp amplification product was obtained which was inserted into pCR™ and used as a hybridization probe in a two-step procedure to isolate corresponding full-size cDNAs. Screening of 5×10^5 p.f.u. of a λgt11-based spinach cDNA (Tittgen *et al.*, 1986) yielded two signals. The corresponding recombinant phages were purified by three replating cycles and shown to contain insert sizes of 0.8 and 1.4 kb. None of them contained the entire coding region of the protein. Screening in a second cycle using a 260 bp PCR-amplified 5′-terminal fragment as a probe resulted in the identification of eight additional recombinant phages. The two largest inserts analysed, 1548 and 1532 bp, were identical in sequence and encoded only one open reading frame (ORF) of 1350 bp (DDBJ/EMBL/GenBank accession No. Y12071). This ORF contained all the four peptide sequences determined from the purified protein (Figure 2). The largest insert was flanked by a 36 bp 5′-untranslated and a 161 bp 3′-untranslated region. The protein starts at the ATG codon at position 37. It has a predicted mass of 49.8 kDa and is translated as a precursor, since the determined N-terminus of the 345 residue polypeptide is preceded by a relatively long, 104 amino acid presequence (Figure 2). The calculated size of the mature protein is 38.2 kDa, consistent with the electrophoretic estimate. The 3′-untranslated region following the translational termination codon, TAG, contains the sequence AATTCA (residues 1506–1511), 44 nucleotides upstream of the cDNA end, that may be involved in post-translational adenylation (Lütcke *et al.*, 1987).

Import and membrane topological studies—lumenal location of TLP40

Hydropathy analysis of the deduced amino acid sequence suggested a predominantly hydrophilic, not intrinsic protein, with a calculated pI of 4.5. Its presequence displayed all attributes of bipartite transit peptides typical for lumenal and bitopic thylakoid proteins (von Heijne *et al.*, 1989; Bartling *et al.*, 1990). It starts with a hydrophilic serine-, leucine- and arginine-rich import sequence (Figure 2) and contains a hydrophobic domain (residues 69–88) close to its C-terminus. This domain is preceded by charged residues (seven positive and three negative charges) and a putative β-sheet at residues 50–54, which in other precursors houses the intermediate cleavage site. It is followed by helix-breaking and turn-inducing residues (Clausmeyer *et al.*, 1993) as well as by a terminal cleavage site with small side chain residues at –1 and –3, most likely D-X-A (von Heijne *et al.*, 1989).

In organello import experiments were performed in order to test that the polypeptide is indeed translated as a precursor and that the isolated cDNA contained the entire transit peptide with the basic attributes for intracellular sorting, import into the chloroplast and translocation of the polypeptide into the thylakoid lumen. Following transcription/translation in the presence of [^{35}S]methionine, the labelled TLP40 precursor (Figure 3, lane 1) was incubated with isolated intact chloroplasts. After washing and thermolysin treatment, the chloroplasts were subfractionated into stroma, thylakoid membranes and lumen. Figure 3 illustrates that the labelled precursor was imported into the organelle and processed to the mature size of the protein as judged by its co-migration with the authentic protein in several gel systems. The same results were obtained with serological analyses using an affinity-purified antibody against TLP40 overexpressed in *Escherichia coli*. The soluble stroma was highly depleted of the imported protein (Figure 3, lane 2), which co-purified almost entirely with the thylakoid fraction (lane 3). Occasionally, a weak signal, intermediate in size between the precursor and mature proteins, was detected. The imported protein was resistant to proteolytic digestion of the thylakoids with thermolysin (lane 4) or trypsin, and also was not affected by high salt treatment (lane 5). Upon sonication of the thylakoids, however, the TLP40 protein was released and recovered in the soluble fraction (lane 6). These experimental observations provide strong evidence that TLP40 is located in the lumen of the thylakoids as predicted from the hydropathy analysis. Residual

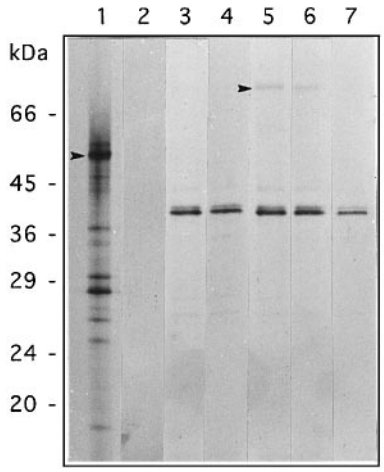

Fig. 3. Import of pre-TLP40 into intact spinach chloroplasts. Pre-TLP40 obtained through *in vitro* transcription/translation was incubated with intact chloroplasts for 30 min. After incubation, samples were thermolysin treated, and the organelles subfractionated. Autoradiogram of a 12% SDS–PAGE. Lane 1: products of translation of pre-TLP40 RNA in a reticulocyte lysate. Lanes 2–4: products found in the chloroplast stroma (2) or in the thylakoid fractions without (3) and with (4) thermolysin treatment. Lane 5: the same as lane 3 but after treatment of thylakoids with 2 M NaBr. Lanes 6 and 7: supernatant and pellet respectively following sonication and centrifugation of thylakoids. The positions of the translation product (lane 1) and putative dimer (lane 5) are indicated by arrowheads. The positions of molecular mass markers are indicated.

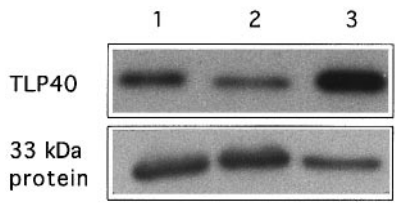

Fig. 4. Heterogeneous location of TLP40 along the thylakoid membrane system. Thylakoids were fragmented by Yeda press, and grana stacks and stroma lamellae vesicles were separated by differential centrifugation. Antibody against TLP40 overexpressed in *E.coli* was used for immunological quantification of its relative amounts in the intact thylakoids (lane 1), grana stacks (lane 2) and stroma-exposed thylakoid regions (lane 3). As a reference, the amount of the *psbO* gene product (33 kDa protein), a subunit of photosystem II enriched in the grana, was analysed in the same fractions.

amounts of the protein were still found to be membrane associated following sonication (lane 7), suggesting that the protein can also interact with the inner thylakoid membrane surface. This possibility was substantiated by the presence of TLP40 in isolated inside-out thylakoid vesicles (not shown).

Gels from the *in organello* assays occasionally displayed an additional faint band at ~80 kDa as exemplified in Figure 3, lanes 5 and 6. This observation could indicate the existence of an SDS-stable dimer or association of TLP40 with another protein of similar size.

Yeda press fragmentation of thylakoids and subsequent differential centrifugation to separate grana stacks and stroma lamellae (Andersson and Anderson, 1980) revealed a pronounced enrichment of TLP40 in the latter fraction as judged by immunological analyses (Figure 4). This is at variance with the fractionation pattern of the *psbO* gene product (Figure 4), the 33 kDa subunit of photosystem II highly enriched in the appressed region of the grana stacks (Andersson and Barber, 1994). It can be concluded, therefore, that the TLP40 has a heterogeneous lateral distribution and is confined mainly to the non-appressed membrane regions while depleted in the grana stack regions of the thylakoid membrane system.

The organelle-imported TLP40 and its stromal processing intermediate appear usually in two bands with slightly different electrophoretic mobility (Figure 3). Preliminary experiments suggest that this doublet could be due to post-translational phosphorylation, a possibility that currently is under investigation.

Sequence and structural analysis of TLP40—an immunophilin-like protein with unique features

The most recent protein databases were searched with the TLP40 polypeptide sequence as the query sequence using the BLASTP program at NCBI www-service. Only the two hypothetical proteins of cyanobacteria mentioned above gave a high homology score, 41 and 46% identity, with the whole length sequence of mature TLP40. Refinement of local similarity analysis and secondary structure predictions revealed a complex domain structure of TLP40 (Figure 5A). As the most striking feature, its C-terminal domain (residues 267–449) was found to resemble proteins of the cyclophilin family predicting a PPIase activity. The N-terminal domain of the protein is unique in that it contains several typical elements for protein–protein interaction, which may be of structural and functional significance (Figure 5A).

Sequence alignment of the C-terminal domain of TLP40 with catalytic domains of other putative PPIases from *Synechocystis* sp., *Arabidopsis thaliana* chloroplast stroma cyclophilin, *Escherichia coli* periplasmic cyclophilin and human cyclophilin A is presented in Figure 5B. The sequence of the putative PPIase from *Synechocystis* sp. displayed the highest homology of all the compared sequences including that of the chloroplast stroma cyclophilin from *Arabidopsis*. The alignment of the TLP40 sequence with that of human cyclophilin A, often used as a basic reference sequence for this class of enzymes, includes three insertions and six deletions, which gives ~25% of residue identity. It is noteworthy that this is the lowest among all known cyclophilins. Stretches of the human cyclophilin A underlined by asterisks in Figure 5B highlight the five-element fingerprint sequence motifs characteristic of cyclophilins derived from comparison of 43 sequences (NCBI, CSAPPISMASE annotated domains). These five motifs, although degenerated, are still recognizable in TLP40 (see Discussion). The motif IV, Q110–D122 in the human sequence, is the least conserved fingerprint sequence. This part of the sequence has been established by structural analyses to account for high affinity binding of the drug CsA (EMBL/SwissProt). The absence of the tryptophan residue, corresponding to W120 in human cyclophilin A, in the *E.coli* periplasmic cyclophilin (Figure 5B) abolishes the sensitivity of the PPIase activity to CsA (Liu and Walsh, 1990). This residue is also absent in TLP40 (Figure 5B).

The N-terminal domain of TLP40 comprises three intriguing structural features (Figure 5A). First, the sequence from L123 to I173 contains repeated leucines or isoleucines at every seventh position (Figure 2), which is a characteristic of the leucine zipper consensus motif

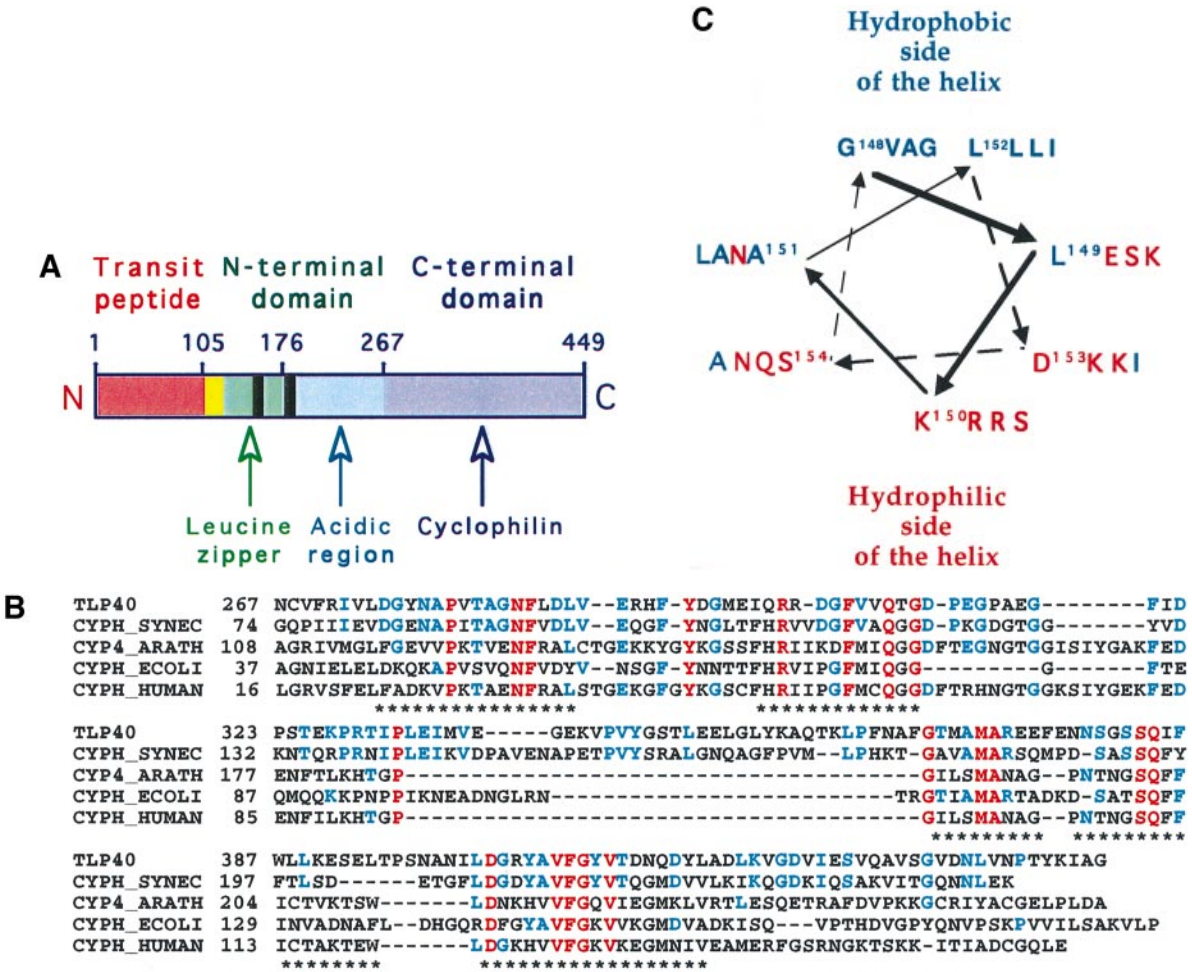

Fig. 5. Sequence analysis of TLP40. (**A**) Overall domain organization of TLP40. The black bars denote the putative phosphatase-binding stretches (see also Figure 2). (**B**) Sequence alignment of the C-terminal domain of TLP40 with putative PPIases of *Synechocystis* sp. (D90900), *A.thaliana* chloroplast stroma cyclophilin (P34791), *E.coli* periplasmic cyclophilin (P20752) and human cyclophilin A (P05092). Alignment was performed by MAP algorithm using www BCM Search Launcher. Five-element fingerprint motifs for cyclophilins are underlined by asterisks in the human cyclophilin A. The residues identical in all five sequences are highlighted in red. Residues in the TLP40 sequence identical to those in at least one of the other compared sequence are indicated in blue. (**C**) Helical wheel projection of a part of the leucine zipper consensus from residue Gly148 to Gly176 of the N-terminal domain of TLP40.

in coiled-coil proteins (Harbury *et al.*, 1993; Lupas, 1996). For TLP40, either two regions of four heptads or a long coil of nine heptads with a skip residue (Lupas, 1996) in the fifth heptad (from L144 to L152) could be considered as structural alternatives. In an α-helical conformation, the residues of the consensus region form a hydrophobic surface with the Leu/Ile on one side of the helix and a hydrophilic surface on the other, as illustrated in Figure 5C. The hydrophobic and hydrophilic regions potentially could provide the site of interaction with other leucine zipper- or coiled-coil-containing proteins (Lupas, 1996).

A second conspicuous feature of the N-terminal segment between the leucine zipper and the cyclophilin domain (E179 to D266) are clustered charged residues (Figure 5A) with a net surplus of acidic residues (19 acidic, 13 basic). In other high molecular weight immunophilins, such as Cyp-40 and FKBP52, corresponding charged sequences were shown to be involved in the binding to other proteins, such as in the interaction with Hsp90 (Ratajczak and Carrello, 1996). Thirdly, the two short stretches GLKALDSVERN[158] and AGLAESKKDRG[185] in TLP40 (Figures 2 and 5A) possess ~60% similarity to the sequence GKKFDSSRDRN[43] in FKBP12, which has been shown to participate in the binding to the protein phosphatase calcineurin (Aldape *et al.*, 1992).

Biochemical evidence for a peptidyl-prolyl isomerase activity of TLP40

The similarity of the C-terminal domain of TLP40 to PPIases of the cyclophilin family raised the question of whether this thylakoid protein indeed possesses this enzymatic activity. To settle that point, a conventional PPIase assay (Fischer *et al.*, 1989) was employed, in a coupled reaction involving the purified TLP40, chymotrypsin and the oligopeptide substrate *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide (Figure 6). Chymotrypsin is capable of hydrolysis of the *trans* but not of the *cis* isoform of the peptide substrate. This hydrolysis releases *p*-nitroaniline, leading to a concomitant absorption increase. As illustrated in Figure 6, the rate of hydrolysis of the peptide substrate in the absence of TLP40 is rather slow, typical of the uncatalysed isomerization process. Addition of purified enzyme to the reaction mixture resulted in a pronounced

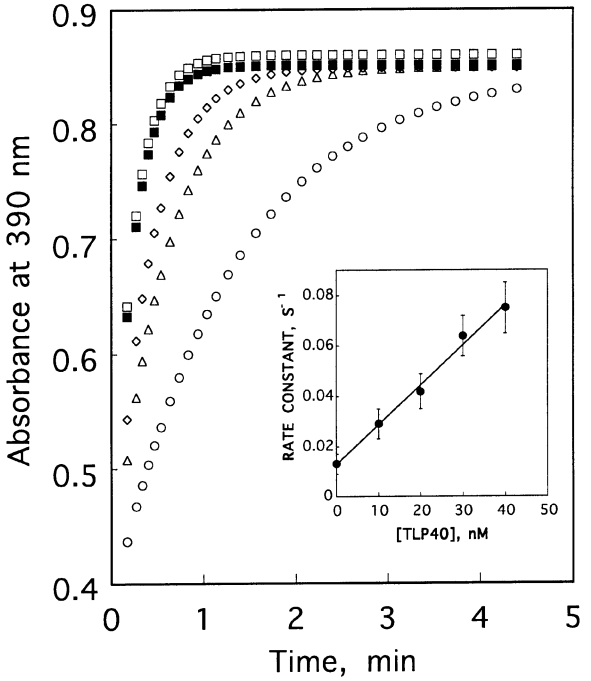

Fig. 6. Enzymatic measurements of the PPIase activity of the purified TLP40. Hydrolysis of the peptide *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide by chymotrypsin in the absence (○) and presence of TLP40 at 10 nM (△), 20 nM (◇) and 40 nM concentrations (□ in the absence and ■ the presence of 10 μM cyclosporin A). The background absorbancy of *p*-nitroaniline release is due to the initial presence of the *trans* form of the PPIase substrate. The insert shows the dependence of the isomerization rate constant on the TLP40 concentration.

acceleration of the absorbance change. Thus, at 40 nM TLP40, the isomerization was completed in <40 s.

To estimate the catalytic efficiency of TLP40, the acceleration of the isomerization rate at different concentrations of the purified enzyme was measured (Figure 6). Calculation of the rate constant increase over the background level in the uncatalysed isomerization (Figure 6, insert) gave a k_{cat}/K_m value of 1.6 ± 0.4/μM/s. This catalytic efficiency is lower than that of the human, bovine and *E.coli* cyclophilins but higher compared with that of the FKBP PPIases (Harrison and Stein, 1990; Liu and Walsh, 1990; Kofron *et al.*, 1991).

The cyclophilin family of PPIases have been designated on the basis of their high-affinity binding of the immunosuppressive CsA with the concomitant inhibition of their activity, normally at nanomolar concentrations of the drug. Thus, the PPIase activity of TLP40 was assayed in the presence of CsA at varying concentrations from 1 nM to 10 μM. No inhibition of the TLP40 PPIase activity was found (Figure 6), consistent with the structural similarity to the *E.coli* enzyme (see above) and in line with the high degeneration of the TLP40 cyclophilin fingerprint motif IV (Q110–D122) compared with the human sequence (see Figure 5B).

TLP40 influences thylakoid protein dephosphorylation

In mammalian cells, the immunophilin-sensitive action on the protein phosphatase calcineurin is the result of blocking the active site of the phosphatase by an immunophilin–immunosuppressive drug complex (see Schreiber, 1992). The sequence indications for potential phosphatase-binding sites of TLP40 and its obstinate co-purification with a thylakoid phosphatase activity suggested an analogy with the case of calcineurin. Since the insensitivity of TLP40 to CsA precluded the possibility of using this drug to investigate its effects on thylakoid protein dephosphorylation, an alternative approach was designed based on the influence of a PPIase substrate peptide on the degree of thylakoid protein dephosphorylation. To perform this experiment, thylakoids were first incubated with ^{32}P-labelled ATP in the light to label membrane phosphoproteins via the light-activated protein kinase activity (Gal *et al.*, 1997). The subsequent dephosphorylation of the phosphoproteins, LHCII as well as the D1 and D2 proteins of the photosystem II reaction centre, was then assayed by incubation of the labelled thylakoids for different periods in the absence or presence of the PPIase substrate *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide. The dephosphorylation assay was performed with both intact thylakoids and thylakoids solubilized by *N*-dodecyl-β-D-maltoside. The latter material was included to ensure the access of the substrate oligopeptide to TLP40 in the thylakoid lumen. When the *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide was added to intact thylakoid membranes, no effect on the dephosphorylation process was observed (Table I). In contrast, in solubilized thylakoids, the presence of the peptide substrate resulted in a dramatic (300%) decrease in the dephosphorylation rate for the LHCII, D1 and D2 phosphoproteins (Table I). Control oligopeptides lacking proline residues were found to exert no effect on the dephosphorylation (not shown). Collectively, these observations suggest that TLP40 functionally interacts with a thylakoid protein phosphatase. Furthermore, this interaction can occur in the absence of an exogenous immunosuppressive drug, at variance with the case of calcineurin in mammalian tissue while similar to the situation found in yeast (Cardenas *et al.*, 1994).

Table I. Influence of the PPIase peptide substrate on dephosphorylation of thylakoid phosphoproteins

	Dephosphorylation half-time (min)	
	LHCII	D1 and D2
Intact thylakoids, no peptide added	19 ± 4	55 ± 8
Intact thylakoids plus the peptide	19 ± 4	57 ± 8
Solubilized thylakoids, no peptide added	37 ± 10	46 ± 12
Solubilized thylakoids plus the peptide	123 ± 15	138 ± 16

To substantiate further the existence of such a cyclophilin–phosphatase interaction as suggested by the chromatographic co-purification (Figure 1) and the functional measurements (Table I), an immunoprecipitation experiment was performed using an immobilized antibody against overexpressed TLP40 which was incubated with solubilized thylakoids. The insert in Figure 7 shows that the antibody precipitated a substantial amount of TLP40 from the lysate (lane 2), in contrast to the control serum (lane 3). Likewise, the phosphatase activity, measured with radioactively labelled phosphopeptides as substrates, was precipitated significantly by the TLP40 antibody but not by the control serum (Figure 7).

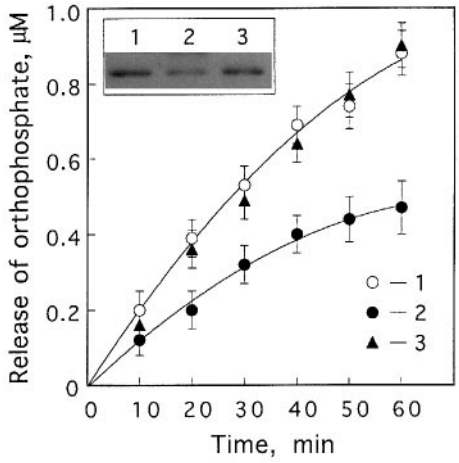

Fig. 7. Co-immunoprecipitation of protein phosphatase activity with TLP40. The phosphatase activity, assayed with 10 μM radioactively labelled phosphopeptides, and the amount of TLP40, measured by Western blot analysis (insert), were determined in the supernatants of solubilized thylakoids before (1) or after immunoprecipitation with an immobilized antibody against TLP40 (2) or control antiserum (3).

Expression patterns of TLP40

In order to gain further information concerning the novel TLP40 protein, the expression of its gene under various conditions was investigated. Hybridization of the pBSC insert to total RNA prepared from spinach and *Arabidopsis* leaf tissue detected only a single, moderately abundant RNA species of 1.5 kb (approximately the size of the largest cDNAs isolated), indicating that the gene is only weakly expressed or that its mRNA concentrations are kept low. In several plant species tested, including tobacco, tomato, rice, barley and *A.thaliana*, TLP40 was found to originate from a single copy gene as well (not shown).

The RNA of TLP40 can be detected in etiolated material. Its steady-state concentrations increase only moderately with illumination, ~2- to 3-fold after 20 h (Figure 8). Western analyses using the antisera elicited against the TLP40 protein recognized the double band at 40 kDa in etiolated tissue in the same position as in green tissue (Figure 8). It is noteworthy that the changes in RNA and protein abundance during greening appeared to parallel each other.

Discussion

The novel chloroplast immunophilin-like protein appears as a new member of the class of 'auxiliary' components involved in photosynthesis. The identification of TLP40 not only provides the first evidence of a complex immunophilin in plants and uncovers the presence of a protein folding catalyst in the thylakoid lumen, but it also provides unexpected insights into the regulation of light-mediated phosphorylation of photosynthetic membrane proteins.

The TLP40 polypeptide originates from a single copy gene in spinach as well as in several other tested plant species. The genome of the cyanobacterium *Synechocystis 6803* (Kaneko *et al.*, 1996) appears to possess an equivalent gene, indicating the conserved nature of TLP40 throughout evolution. Several lines of evidence established that the isolated cDNA encodes the correct, biochemically identified polypeptide and its complete coding region: the cDNA

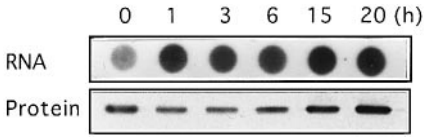

Fig. 8. Parallel accumulation of TLP40 RNA and protein during greening of etiolated spinach seedlings as revealed by Northern and Western blot analysis respectively. For the dot-blot Northern analysis (autoradiogram, upper panel), 2 μg of polyadenylated RNA isolated from etiolated (0 h) material or after 1, 3, 6, 15 and 20 h of illumination were spotted onto a Pall Biodyne nylon membrane and hybridized with a ^{32}P-labelled TLP40 cDNA probe.

contained all oligopeptide sequences determined, the import competence of the *in vitro* synthesized translation product, the serology and finally the electrophoretic co-migration of the processed polypeptide in the *in organello* assays with authentic TLP40.

The existence of a bipartite transit sequence, the *in organello* import experiments and membrane topological studies demonstrate unequivocally that TLP40 is located in the thylakoid lumen. Furthermore, the protein was found to be located in the stroma-exposed thylakoid regions. This lateral heterogeneity suggests not only that TLP40 should be considered as a freely diffusable lumen component but that it can interact with the inner surface of the stroma-exposed thylakoid regions, at least temporarily. As will be discussed below, this interaction may be of functional significance. Electrophoresis in non-denaturing gels combined with Western analysis indicates that a minor fraction of the protein can exist in either homo- or heterodimeric form.

The finding that TLP40 is located in the thylakoid lumen suggests that the significance of this chloroplast compartment has been greatly underestimated from both a compositional and a functional point of view, although recent discoveries demonstrating the presence of polyphenol oxidase (Sokolenko *et al.*, 1995), zexanthin epoxidase (Marin *et al.*, 1996) and violaxanthin deepoxidase (Bugos and Yamamoto, 1996) have increased interest in this chloroplast compartment. This is in line with more recent data showing that a preparation of pure thylakoid lumen contained at least 25 different polypeptides including TLP40 (Kieselbach *et al.*, 1998). One likely role of TLP40 may therefore be to catalyse isomerization of proteins translocated across the thylakoid membrane into the lumen. TLP40 consequently would serve as a lumenal counterpart of the SecA/B system which contributes to protein unfolding for transport of polypeptides from the opposite stromally exposed membrane side (see Schatz and Dobberstein, 1996). Consistent with this role is its location predominantly in the stroma-exposed thylakoid regions, the major integration site of newly synthesized or imported polypeptides (Cohen *et al.*, 1995), and the spatial and temporal expression pattern of its gene. In contrast to the stroma-located chloroplast immunophilins (Luan *et al.*, 1994a,b), TLP40 is also present in etiolated material (Figure 8), indicating that it may also be essential for the early stages of plastid development.

The presence of TLP40 in the thylakoid lumen raises a general question with respect to the intracellular location of immunophilins. These enzymes are known to cooperate with other components, i.e. chaperones, in energy-requiring processes, and consequently are assumed to be absent

in compartments lacking prerequisites for ATP hydrolysis, such as the mitochondrial intermembrane space. There currently is no evidence for the presence of ATP or any form of ATPase activity in the thylakoid lumen. Therefore, at first glance, the identification of TLP40 argues against an obligate connection between immunophilins and ATP-dependent processes, a notion that is also supported by the existence of a cyclophilin in the periplasmic space of *E.coli* (Liu and Walsh, 1990). On the other hand, the recent observation of a chaperone located in the thylakoid lumen (Schlicher and Soll, 1996) and nucleotide binding to the PsbP protein at the inner thylakoid surface (Gal *et al.*, 1996) may call for further re-examination of the presence of ATP in the thylakoid lumen.

Compared with the human type A cyclophilin, TLP40 shows the lowest degree of identity (25%) to any known cyclophilin. This low conservation of TLP40 combined with its enzymatic ability may provide a clue to delineate the minimal structural requirements for PPIase activity. Furthermore, the insensitivity towards CsA distinguishes TLP40 from most other cyclophilins except for the cyclophilin of *E.coli* periplasmic space (Liu and Walsh, 1990). In this context, it is relevant to note that TLP40 shares a degeneration of cyclophilin fingerprint motif IV with this *E.coli* enzyme (Figure 5).

The majority of PPIases identified so far are 10–20 kDa in size (see Rudd *et al.*, 1995; Marks, 1996), including those of the chloroplast stroma (Lippuner *et al.*, 1994; Luan *et al.*, 1994a,b). The few larger immunophilins, to which TLP40 can now be added as the first plant member, possess additional domains of structural and functional significance. In the case of TLP40 (Figure 5A), there is an acidic region and a predicted amphiphilic leucine zipper which may be important for the oligomerization and/or interaction of the immunophilin with the thylakoid inner membrane surface. The charged segments of the N-terminal region contain stretches (Figure 2) that resemble epitopes of other immunophilins known to participate in the binding to the protein phosphatase calcineurin (Aldape *et al.*, 1992). The chromatographic co-purification and the immunological co-precipitation of TLP40 with a phosphatase activity combined with compelling evidence for a functional link between this immunophilin and thylakoid protein dephosphorylation (Table I) represent a highly intriguing analogy to the case of calcineurin. Thus, our data provide a second case, where an immunophilin–phosphatase interaction can regulate an entirely different biological process such as photosynthetic energy conversion. However, our observations also point to some important distinctive aspects with respect to the regulatory effects by TLP40, since it does not require the presence of an immunosuppressive drug. The only previous example of an immunophilin–calcineurin interaction in the absence of immunosuppressive ligands was reported in yeast (Cardenas *et al.*, 1994). Furthermore, the location of TLP40 in the lumen compartment argues against a direct interaction between the immunophilin and the phosphatase active site exposed at the outer thylakoid surface. This is supported by the lack of any effect by the PPIase substrate when added to intact thylakoid membranes (Table I). The regulatory effect on thylakoid protein dephosphorylation should rather require a transmembrane signalling probably involving an interaction between TLP40 in the lumen and a transmembrane receptor protein, in the simplest case with an intrinsic protein phosphatase. Such a transmembrane signalling would be in analogy with the redox-dependent activation of the stroma-exposed thylakoid protein kinase which requires the binding of plastoquinol at the Qo-site of cytochrome *b/f* facing the lumenal side of the membrane (Vener *et al.*, 1997). Our data suggest a hitherto unknown control mechanism of thylakoid dephosphorylation, arguing against previous concepts that favoured an unregulated process (see Gal *et al.*, 1997). The exact physiological significance of the immunophilin-dependent transmembrane regulation of protein dephosphorylation via the lumen remains to be established. However, recent data on the repair of photoinhibitory damage to photosystem II has demonstrated that turnover of the D1 reaction centre protein and reversible protein phosphorylation are linked (see Andersson and Aro, 1997), thereby suggesting a possible role for TLP40 in light of its dual functionality as a folding catalyst and regulator of dephosphorylation.

Various findings have demonstrated that transcription and steady-state RNA concentrations are usually not the rate-limiting steps in the decoding of structural components for the photosynthetic machinery. The light-induced biogenesis of the thylakoid system is generally characterized by relatively uniform changes of mRNA levels for almost all components studied, but RNA and protein patterns frequently are discordant (see Herrmann, 1996). This appears to be different in the case of TLP40 for which changes in the low abundance steady-state and protein levels coincided (Figure 8), indicating that regulatory components may follow expression schemes different from those of genes encoding structural components.

In conclusion, the discovery of TLP40 provides the first evidence for a direct involvement of an immunophilin in an autotrophic process and the presence of complex immunophilins in plants. More specifically, it has provided new information with respect to both regulation of light-mediated protein phosphorylation and biogenesis of thylakoid proteins, as well as stressing the importance of the 'neglected' thylakoid lumen as a cellular compartment of functional significance.

Materials and methods

Isolation of intact chloroplasts; chloroplast subfractionation; protein extraction

Intact spinach chloroplasts isolated as described in Bartlett *et al.* (1982) were pelleted and lysed in 10 mM HEPES-KOH (pH 8.0), 5 mM $MgCl_2$ (LS buffer) at a final concentration of 1 mg chlorophyll/ml determined according to Arnon (1949). After incubation for 10 min on ice, the thylakoids were pelleted at 15 000 *g* for 5 min. Thylakoids were washed twice and finally resuspended in LS buffer to a concentration of 1 mg chlorophyll/ml. Lumenal proteins were released by sonication (Branson Sonifier) of thylakoids with 7 s maximum energy bursts with interspersed cooling intervals. Soluble proteins were collected after 1 h centrifugation at 100 000 *g* and 4°C.

Isolated thylakoids, suspended in 10 mM Na-phosphate (pH 7.4), 5 mM NaCl, 5 mM $MgCl_2$ and 100 mM sucrose, were fragmented by two passages through a Yeda press at a nitrogen pressure of 10 MPa (Andersson and Anderson, 1980). The homogenate was subjected to differential centrifugation; 10 000 *g* for 30 min (grana stack fraction), 40 000 *g* for 30 min and 100 000 *g* for 60 min (stroma-exposed thylakoid fraction). Inside-out thylakoid vesicles were isolated after further fragmentation by Yeda press followed by aqueous polymer two-phase partition (Andersson and Anderson, 1980).

Extracts of denatured proteins were prepared by incubation for 10 min at 80°C of homogenized frozen leaf and etiolated seedling tissue thawed

in 0.5 M Tris–HCl (pH 6.8), 50 mM EDTA, 1% SDS and 0.07% β-mercaptoethanol. The samples were centrifuged at 25 000 *g* for 10 min at 25°C and protein concentrations measured according to Bradford (1976) with bovine serum albumin (BSA) dissolved in the same buffer as a standard.

Protein purification

Thylakoid membranes were isolated from 300 g of 6-week-old spinach leaves according to Andersson *et al.* (1976) and washed with 2 M NaBr as described by Hurt and Hauska (1981) to remove peripheral proteins. The membranes were solubilized by Triton X-100 (1%/mg of chlorophyll) at 4°C for 30 min and centrifuged at 100 000 *g* for 1 h. The supernatant was loaded onto a Sepharose Q column (15 ml), equilibrated with 0.5% Triton X-100 in 20 mM Tricine-NaOH (pH 7.8). After washing with 0.1 M NaCl in the same buffer, a 25 ml fraction eluted with 0.3 M NaCl was collected. Concentration and desalting of this fraction were followed by three consecutive FPLC runs on a Resource Q column (1 ml) using a 0–0.5 M NaCl gradient in 20 mM Tricine-NaOH (pH 7.8) sequentially with 0.5% Triton X-100, 2 mM *N*-dodecyl-β-D-maltoside or 8 mM CHAPS. Fractions eluted at 0.23–0.27 M NaCl were collected for further purification. The final chromatographic fractions were concentrated to 0.1 ml and subjected to FPLC on a Superose 12 (HR 10/30) column equilibrated with 50 mM imidazole-HCl (pH 6.5), containing 2 mM *N*-dodecyl-β-D-maltoside.

Protein analysis and microsequencing

Polypeptide analyses were carried out by SDS–PAGE (Laemmli, 1970), generally using 15% acrylamide gels. N-terminal protein sequencing was performed from PVDF membranes according to Matsuradaria (1987) by Edman degradation and pulse liquid phase sequencing using an Applied Biosystems sequenator ABS 477A. To determine internal sequences, the dried gel band containing the 40 kDa protein was excised, subjected to cyanogen bromide cleavage (Takeya *et al.*, 1982) and loaded onto a 18.75% SDS–PAGE containing 6 M urea (Kadenbach *et al.*, 1983). The peptides were separated by electrophoresis, electroblotted onto PVDF membrane, excised and used for microsequencing.

Western analyses of TLP40 were performed using polyclonal antiserum (at dilutions 1/1000–1/2000) raised against the protein overexpressed in *E.coli*. Protein–antibody conjugates were visualized by chemiluminescence (Amersham, Braunschweig, Germany) or alkaline phosphatase reaction.

PCR-mediated isolation of TLP40 cDNA

Total DNA was prepared from lysates of ~1.7×10^8 amplified recombinant phages of a λgt11-based cDNA library (Tittgen *et al.*, 1986) using standard PEG/NaCl precipitation of lambda particles (Sambrook *et al.*, 1989) followed by a phenolization step and ethanol precipitation.

The N-terminal and three internal peptide sequences were used for the construction of a set of seven weakly degenerated oligonucleotide primers representing all possible peptide orientations with respect to each other (5′-ATHAARGAYCCIGARGC-3′ from the N-terminal sequence, 5′-ATGGARATHCARMGICG-3′ and 5′-CGISKYTGDAT-YTCCAT-3′ from one of the internal peptide sequences, 5′-ATGGTIG-ARGGIGARAA-3′ and 5′-TACCAICTYGCICTYTT-3′ from the second one, and 5′-GARGARTTYGARAAY-3′ and 5′-KAARTTYTCRAAYT-CYTC-3′ from the third internal peptide). The oligonucleotides were obtained from MWG-BIOTECH (Ebersberg, Germany). Thirty-cycle PCR amplifications with 1.5 min elongation time were performed using different combinations of primers and annealing temperatures with 10 ng of isolated template DNA under the following conditions: 100 pM each primer, 0.2 mM dNTP and 1.5 mM $MgCl_2$. A single 693 bp PCR product obtained with the N-terminal primer and primer 5′-TACCAICTYCCIC-TYTT-3′ derived from one of the internal peptides was inserted into pCR™ II (Invitrogen), cloned into *E.coli* and sequenced with Sequenase version 2.0 (U.S. Biochemicals). This recombinant DNA was designated pCR700.

The randomly primed (MBI, Vilnius, Lithuania) [^{32}P]dCTP-labelled *Eco*RI insert from pCR700 was used as a probe for rescreening the λgt11 cDNA library. Hybridization was performed according to Church and Gilbert (1984), in 500 mM Na_2HPO_4 (pH 7.2), 7% SDS, 1% BSA, 1 mM EDTA buffer at 64°C for 15 h followed by stringent washing. Positive phages were plaque purified, and the inserts were recloned into pBSC (Stratagene, San Diego) and sequenced.

DNA and RNA analysis

Total DNA from *Spinacea oleracea*, as well as from *Nicotiana tabacum*, *Solanum lycopersicum*, *Oryza sativa* and *Hordeum vulgare*, was isolated according to Rether *et al.* (1993), digested with *Bam*HI, separated in a 1% agarose slab gel and transferred to Pall Biodyne nylon membrane (Pall, Dreieich, Germany). Hybridization with [^{32}P]dCTP-labelled 1.5 kb *Eco*RI insert from a plasmid pBHF1 containing the entire cDNA was performed as described above. Gene copy number was determined from signal intensity to the respective C-values using a plasmid-based copy number determination.

Total spinach and *Arabidopsis* RNA was isolated from green leaf tissue according to de Vries *et al.* (1988), resolved on a 1.2% formaldehyde-containing agarose gel and blotted onto nylon membrane. Northern analysis was performed using the [^{32}P]dCTP-labelled 800 bp *Bsb*I–*Eco*RI fragment of pBHF1 as a probe.

Polyadenylated RNA was isolated from de-etiolated spinach seedlings illuminated for 0, 1, 3, 6, 15 and 20 h (light intensity 50 μmol/m^2/s) respectively, as described in Pötter and Kloppstech (1993) and in the manufacturer's procedure (Pharmacia, Freiburg i. Br., Germany) using oligo(dT)–cellulose chromatography. Equal amounts of poly(A)$^+$ RNA were used for dot-blot transfer and hybridization with ^{32}P-labelled 1.5 kb *Eco*RI cDNA insert. Signals linear in intensity with exposure time were scanned using a Fuji BAS-1500 phosphoimager and quantified with the TINA 2.09g software package (Raytest, Straubenhardt, Germany).

Import of TLP40 precursor into isolated spinach chloroplasts

pBHF1 plasmid containing the coding region of the TRL40 precursor was linearized with *Kpn*I downstream of the inserted cDNA fragment. The transcription reaction was performed *in vitro* with T7 RNA polymerase. Transcript was then translated for 1 h at 30°C in a cell-free reticulocyte lysate in the presence of [^{35}S]methionine, both purchased from Amersham (Braunschweig, Germany).

Import into the intact chloroplasts was performed in the light (50 μmol/m^2/s) for 30 min at 25°C in a total volume of 240 μl containing 30 μl of translation assay and chloroplasts corresponding to 100 μg of chlorophyll. [^{32}P]phosphoglycerate/oxalacetate-supported assays were performed. The chloroplasts were protease treated, washed and subsequently subfractionated into stroma and thylakoids following osmotic shock according to Clausmeyer *et al.* (1993). Soluble lumenal components were released from intact thylakoids by sonication as described above. To monitor for possible membrane association of TLP40, thylakoids were incubated on ice in the presence of 2 M NaBr. Proteins from the various subfractions were then precipitated by acetone, pelleted at 13 000 *g* for 2 min and dissolved in sample buffer (Laemmli, 1970) for electrophoresis.

Peptidyl prolyl isomerase (PPIase) assay

PPIase activity was assayed using a coupled reaction involving chymotrypsin (Fischer *et al.*, 1989). The peptide substrate *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide (Calbiochem) was dissolved up to 5–7 mM in 470 mM LiCl in trifluoroethanol (Kofron *et al.*, 1991) to increase the population of the *cis* isomer to 45–55%. The reaction was started by addition of the peptide substrate (50–70 μM) supplied with 30–50 μM chymotrypsin and 0.01–0.08 μM of protein sample in an final volume of 1 ml of Tricine-NaOH (pH 7.8), at 10°C. The absorbance change at 390 nm due to release of *p*-nitroaniline was recorded using a Shimadzu UV-3000 spectrophotometer. The k_{cat}/K_m values for the isomerization catalysis were calculated according to the equation $k_{cat}/K_m = (k_{obs} - k_u)/[PPI]$ (Harrison and Stein, 1990), where k_{obs} represents the first-order rate constant for isomerization in the presence of PPIase, and k_u the first-order rate constant for the uncatalysed spontaneous isomerization. The molar concentration of TLP40 was calculated using M_r = 40 000 and determination of protein according to Bradford (1976); where indicated, TLP40 was pre-incubated with 1 nM–10 μM CsA (Calbiochem) for 15–60 min before measuring enzyme activity.

Assay of protein dephosphorylation in thylakoid membranes

Thylakoids (0.5 mg chlorophyll/ml) were phosphorylated in the light (200 μmol photons/m^2/s) for 20 min by [γ-^{32}P]ATP (0.1 mM, 50 μCi/ml) in a buffer containing 50 mM Tricine (pH 7.8), 100 mM sorbitol, 5 mM $MgCl_2$ and 10 mM NaF. The excess of low molecular weight radioactivity was removed by washing of the membranes with the same buffer without NaF. To assay protein dephosphorylation, the labelled thylakoids, intact or solubilized by 5 mM *N*-dodecyl-β-D-maltoside, were incubated for 15–180 min at room temperature in the absence or presence of 4 mM *N*-succ-Ala-Ala-Pro-Phe-*p*-nitroanilide. Reactions were terminated by addition of electrophoresis sample buffer. Following SDS–PAGE, the gels were stained with Coomassie brilliant blue, dried and autoradiographed using X-ray film. Quantification of the

protein phosphorylation level was done by scanning the autoradiograms by laser densitometry using the software package Image Quant from Molecular Dynamics.

Co-immunoprecipitation of the phosphatase activity with TLP40

For immobilization of antibodies, 10 µl of rabbit antiserum against TLP40 or a control serum was agitated with 20 µl of protein A–Sepharose in water (1:1) for 3 h followed by triple washings of the beads in 50 mM Bis-Tris (pH 6.9) 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT) and 20 mM *N*-dodecyl-β-D-maltoside (buffer A). Thylakoids, washed with 2 M NaBr, were solubilized in buffer A and centrifuged to remove the insoluble material. Then 100 µl aliquots of the supernatant were mixed with the immobilized antibody and agitated overnight at 4°C. The amount of TLP40 left in the supernatant after the immunoprecipitation was determined by Western blotting. The protein phosphatase activity was assayed with ^{32}P-labelled phosphopeptides obtained by proteolytic cleavage of thylakoids phosphorylated by [γ-^{32}P]ATP according to Andersson *et al.* (1995). The orthophosphate released from the phosphopeptides was determined after acid-molybdate extraction as described in MacKintosh (1993).

Accession number

The TLP40 sequence data have been submitted to the DDBJ/EMBL/GenBank databank under accession No. Y12071.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 184), the Swedish Council for Forestry and Agricultural Research, the Swedish Natural Science Research Council, the Royal Swedish Academy of Sciences and the Human Frontier Science Programme.

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Received October 7, 1997; revised and accepted January 13, 1998