PSI-O, a new 10-kDa subunit of eukaryotic photosystem I

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Abstract A novel polypeptide with an apparent molecular mass of 9 kDa was detected after sodium dodecyl sulphate–polyacrylamide gel electrophoresis of *Arabidopsis* photosystem I (PSI) and was N-terminally sequenced. Corresponding cDNA clones encode a precursor protein of 140 amino acid residues which was imported into isolated intact chloroplasts and processed to the mature protein, designated PSI-O. The mature protein has two transmembrane helices and a calculated mass of 10 104 Da. The PSI-O protein was also shown to be present in PSI isolated from barley and spinach, and was essentially absent in chloroplast grana. Expressed sequences encoding similar proteins are available from many species of plants and green algae.

Key words: *Arabidopsis*; Chloroplast import; Photosynthesis; Photosystem I; *psaO*; Photosystem I-O

1. Introduction

Plant photosystem I (PSI) is a multiprotein complex, consisting of at least 17 different subunits named PSI-A to PSI-L, PSI-N, and the four light-harvesting proteins Lhca1–4 [1]. The 13 PSI core proteins appear to be present in one copy per P700, whereas two copies of the four peripheral antenna proteins seem to be organised as homo- and heterodimers in the light-harvesting complex I (LHCI) [1–3]. Eukaryotic PSI has features that distinguish it from PSI of cyanobacteria. The three subunits PSI-G, PSI-H and PSI-N, and the membrane-embedded LHCI are only found in eukaryotes. In recent years a series of transgenic *Arabidopsis* plants lacking PSI-E, -F, -G, -H, -K, -L, -N, or Lhca4 have been generated to gain more insight into the role of the individual subunits in PSI [4–10]. The role of the different subunits of eukaryotic PSI has recently been reviewed [1].

The primary structure of the plant specific subunit PSI-N was published in 1993 [11]. Since then, no additional subunits have been reported in plant PSI. The *Arabidopsis* mutant lacking the PSI-N subunit [5] enabled the discovery of the protein PSI-O, as described in this paper.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana (L.) Heyhn ecotype Columbia wild type (WT) and the *Arabidopsis* plants lacking PSI-N were grown in compost in a controlled environment *Arabidopsis* chamber (Percival AR-60L, Boone, IA, USA) at a photosynthetic flux of 100–120 μmol photon m^{-2} s^{-1} in an 8 h light/16 h dark cycle, and 70% relative humidity [7,8]. Seedlings of *Pisum sativum* var. Kelvedon Wonder were grown in compost for 8 days at 20 ± 2°C under a photosynthetic flux of 50 μmol photon m^{-2} s^{-1} and a 12 h light/dark cycle.

2.2. Isolation of PSI complexes

PSI complexes were isolated from 6–8-week-old *Arabidopsis* leaves by solubilisation of the thylakoid membranes (1 mg chlorophyll (Chl) ml^{-1}) for 10 min at 0°C with 1% dodecyl-β-maltoside (Sigma) at a detergent/Chl ratio of 10:1, followed by sucrose gradient ultracentifugation as previously described [8,12,13]. Spinach PSI was prepared in the same way, except that decyl-β-maltoside was used.

Highly purified barley PSI was prepared by solubilisation with decyl-β-maltoside and column chromatography as previously described [14]. Barley thylakoids were fractionated into grana and stroma lamellae according to Albertsson et al. [15].

2.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and N-terminal sequencing

For the analysis of their protein composition, the *Arabidopsis* PSI preparations were subjected to 16–23% denaturing SDS–PAGE as described in [13]. Proteins were transferred to a ProBlott membrane (Applied Biosystems, Foster City, CA, USA) following the protocol of the manufacturer and as described in [11]. Coomassie brilliant blue-stained bands containing PSI-O were cut out of the membrane and subjected to amino acid sequencing with a sequenator model 470A coupled to a phenylthiohydantoin analyser model 120A (both from Applied Biosystems).

2.4. Antibody production

A histidine-tagged protein based on cDNA clone 113H17T7 from the *Arabidopsis* Biological Resource Center (Columbus, OH, USA) [16] was expressed in *Escherichia coli*. A *Bam*HI/*Xho*I internal fragment of 277 bp corresponding to the mature protein was amplified by polymerase chain reaction and was cloned in the *Bam*HI/*Xho*I site of PET-41a (+) (Novagen, Bad Soden, Germany). The resulting plasmid was used to transform *E. coli* BL21 (DE3). The fusion protein was present in inclusion bodies, which were purified, solubilised and subjected to Ni-affinity chromatography according to the manufacturer's instructions. The isolated fusion protein was used to immunise a rabbit. Rabbit antiserum was collected after four injections of a total of 1 mg of protein.

2.5. Immunoblot analysis

The proteins of isolated PSI and thylakoid preparations were separated on 8–25% SDS–PAGE [17]. Proteins were transferred to nitrocellulose membranes using a semi-dry electroblotter (Ancos, Ølstykke, Denmark). The polyclonal rabbit antibody raised against PSI-O was detected using an enhanced chemiluminescent detection system (ECL,

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Abbreviations: Chl, chlorophyll; LHC, light-harvesting complex; Lhca, light-harvesting chlorophyll *a*/*b*-protein of photosystem I; Lhcb, light-harvesting chlorophyll *a*/*b*-protein of photosystem II; PS, photosystem; WT, wild type

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Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.6. Import of precursor PSI-O into chloroplasts

Two cDNA clones encoding *PsaO* with EMBL accession numbers AV533754 and AV533966 were obtained from the Kazusa DNA Research Institute, Japan [18] and fully sequenced. Experimental work was continued using clone AV533754. The plasmid was linearised by digestion with *Xho*I and transcribed in vitro using T3 RNA polymerase (Promega). Pre-PSI-O was synthesised by translation of the mRNA template in a wheat germ lysate system (Promega) in the presence of [^{3}H]leucine (Amersham Pharmacia). Intact pea chloroplasts were isolated from seedling leaves as described in [19], and were incubated with pre-PSI-O as described in [20]. Samples were analysed by tricine–SDS–PAGE [21] and fluorography.

3. Results and discussion

3.1. SDS–PAGE resolves a new PSI subunit in Arabidopsis PSI complexes lacking PSI-N

The PSI holo-complex consisting of core and the LHCI subunits can be isolated from *Arabidopsis* WT and mutant thylakoid membranes. Denaturing 16–23% SDS–PAGE resolves at least 15 Coomassie brilliant blue-stained PSI protein bands in PSI preparations from *Arabidopsis* WT (Fig. 1). These are, in descending order, the co-migrating PSI-A and B, Lhca3, -2, -4, -1, two PSI-D bands, PSI-F, -L and -E, and a low molecular mass region with PSI-H, the co-migrating PSI-G and -N, PSI-C and PSI-K. The 4-kDa subunits PSI-I and PSI-J are not well resolved in the gel. The lack of subunit PSI-N in the PSI preparation from the *Arabidopsis* transformant

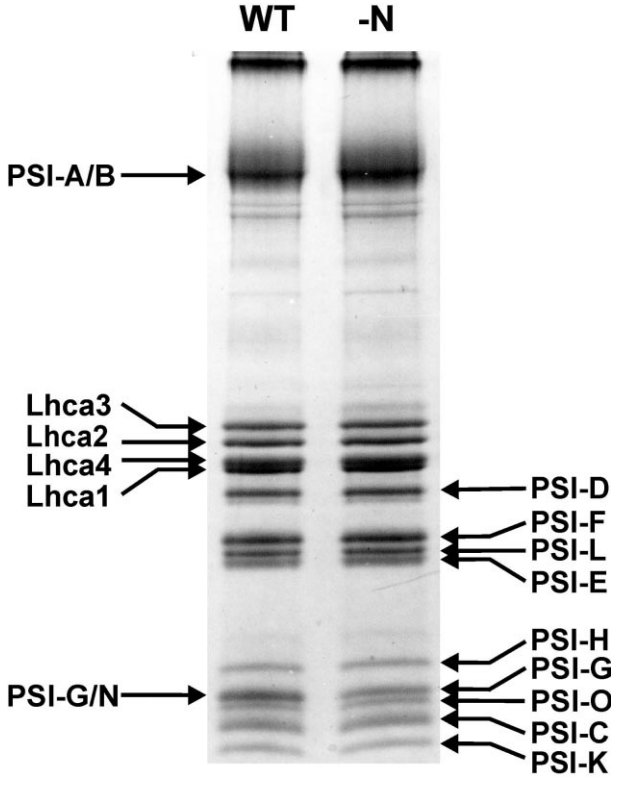

Fig. 1. Polypeptide composition of PSI complexes isolated from *A. thaliana* WT and mutant leaves (-N). The proteins (corresponding to 2.5 μg Chl) were separated by 16–23% gradient SDS–PAGE. The protein bands with an apparent molecular mass of 9 kDa designated PSI-O were subjected to amino acid sequencing.

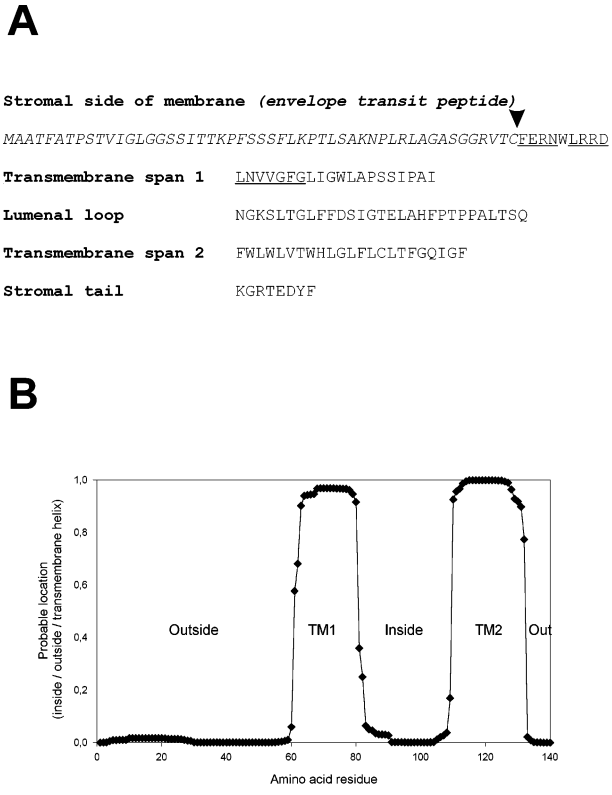

Fig. 2. Amino acid sequence (A) and transmembrane prediction (B) of the precursor protein PSI-O from *Arabidopsis*. The amino acid sequence obtained after N-terminal sequencing of the isolated protein is underlined. The possible maturation site is shown with an arrowhead.

enabled the clear visualisation of an additional protein band with an apparent molecular mass of 9 kDa that is designated PSI-O in Fig. 1. Later, it was even possible to resolve this protein band in PSI from *Arabidopsis* WT after prolonged electrophoresis as shown in Fig. 1.

The PSI proteins from *Arabidopsis* WT and the transformant without PSI-N were transferred onto ProBlott membranes, and the new 9-kDa protein band from both preparations was subjected to N-terminal sequencing. Two to four different amino acid residues could be identified for the first four positions in the two protein samples, with identical residues in both preparations resulting in the amino acid sequence FERN. The amino acid at position five could not be clearly identified, probably due to insufficient amounts of material. Tryptophan, which turned out to be the amino acid residue in this position is one of the more difficult amino acids to identify. For positions 6 to 15, the protein from the *Arabidopsis* plants lacking PSI-N gave a single amino acid signal at each position, resulting in the sequence LRRDLNVVGF. For the WT sample the situation was less clear due to contaminating PSI-N and PSI-G, but all corresponding amino acids were present in considerable amounts.

Based on the N-terminal amino acid sequence, cDNA clones encoding PSI-O were obtained from the Kazusa DNA Research Institute (Yana, Japan) (EMBL: AV533754 and AV533966) [18] and from the *Arabidopsis* Biological Resource Center (Columbus, OH, USA) (113H17T7, EMBL:

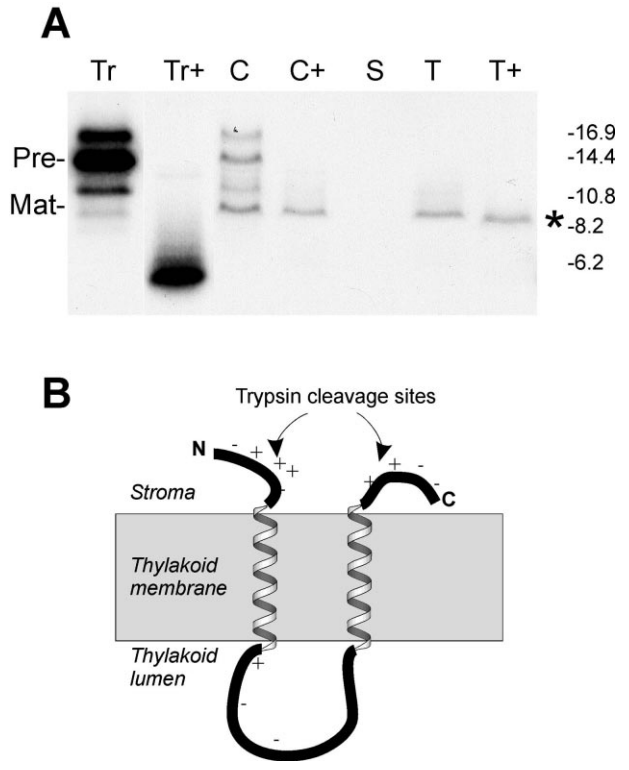

Fig. 3. A: Import of pre-PSI-O into isolated, intact chloroplasts. Intact chloroplasts were isolated from pea seedlings (see Section 2) and incubated with in vitro-translated pre-PSI-O (lane Tr, marked Pre-). After the incubation, the chloroplasts were washed and fractionated to determine the location of imported protein. The lanes are as follows: C: washed, recovered chloroplasts; C+: thermolysin-treated chloroplasts; S: stromal extract; T: thylakoid membranes; T+: trypsin-treated thylakoids; Tr+: translation mixture digested with trypsin. The imported PSI-O protein is marked Mat- and the degradation product associated with the thylakoids after trypsin digestion is marked *. Sizes of molecular mass markers are shown in kDa. Thermolysin and trypsin were both used at final concentrations of 0.2 mg/ml. B: Schematic diagram of the topology and trypsin cleavage sites of PSI-O.

AT4404) [16] and completely sequenced. The genomic sequence is submitted by the *A. thaliana* Genome Center (Philadelphia, PA, USA) with the EMBL accession number AC006932 and specifies a transcript which completely matches the Kazusa cDNA clones. In contrast, the ABRC clone has accumulated a number of minor changes in the part corresponding to the transit peptide.

The open reading frame of 140 codons encodes a precursor protein with a calculated mass of 15 143 Da composed of the mature protein of 89 amino acid residues with a calculated molecular mass of 10 104 Da and a transit peptide consisting of 51 amino acid residues (Fig. 2A). Based on the N-terminal sequence of the PSI-O polypeptide, the transit peptide is processed between Cys^{51} and Phe^{52}. The full open reading frame was analysed using the TMHMM2.0 prediction server available at the Center for Biological Sequence Analysis, Technical University of Denmark [22]. As summarised in Fig. 2B, the mature protein is predicted to contain two transmembrane helices, connected by a lumenal loop of about 30 amino acids. Both N- and C-termini, which are each only about 8 amino acids long, are predicted to lie on the stromal side of the thylakoid membrane.

3.2. The PSI-O precursor is imported into chloroplasts, processed and integrated into the thylakoid membrane

The ability of the N-terminal extension of PSI-O to function as a chloroplast transit peptide was tested in vitro. Intact pea chloroplasts were incubated with in vitro translated, radiolabelled pre-PSI-O according to Section 2. After the incubation, the chloroplasts were washed, then fractionated to determine the location of the imported protein and analysed by tricine–SDS–PAGE and fluorography. Fig. 3A shows that incubation of the precursor protein mixture (lane Tr) with chloroplasts (lane C) gives rise to a labelled band with an apparent molecular mass of 9 kDa, which we assign as the mature PSI-O protein. Digestion of the outside of the chloroplasts with the protease thermolysin (lane C+) removes the unimported protein, whereas the 9 kDa band remains protected and therefore inside the chloroplasts. The labelled protein is associated with the thylakoid membrane fraction (lane T), but is absent from the stromal extract (lane S). After washing the thylakoid membrane fraction and digesting it with trypsin, a degradation product is seen (lane T+, marked with a *). The change in mobility of the labelled protein is consistent with the removal of one or both of the small N- and C-termini, which are predicted to lie on the stromal side of the thylakoid membrane, and thus be accessible to protease. Both termini contain arginine or lysine residues that can be attacked by trypsin. The degradation product does not derive from uninserted protein, because digestion of the translation mixture by trypsin results in the conversion of the labelled protein to very low molecular mass peptides (lane Tr+). These results support the topology predictions for PSI-O, which are summarised in Fig. 3B; in this orientation PSI-O obeys the 'positive inside rule' [23]. Very similar experimental results were obtained when pre-PSI-O was imported into isolated *A. thaliana* chloroplasts (data not shown).

3.3. PSI-O is a protein component of PSI

The PSI-O protein is present in *Arabidopsis* PSI and the intensity of the stained band suggest the presence of about one copy of PSI-O per P700 (Fig. 1). Immunoblotting with the antibody raised against the PSI-O protein shows that the protein is almost absent in the barley mutant *viridis-zb*^{63} (Fig. 4). This mutant has only about 2% active PSI compared to the wild-type, and all the previously known subunits of the PSI core are present at a very low level [24]. All other proteins in

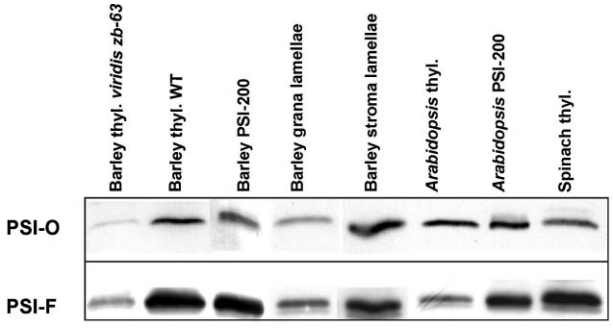

Fig. 4. Immunoblot analysis of thylakoid and PSI preparations from different species. Proteins corresponding to 4 μg of Chl for membrane preparations and 2 μg of Chl for PSI preparations were loaded in each lane, separated by 8–25% SDS–PAGE and blotted onto nitrocellulose membranes. The blot was incubated with antibodies against PSI-O and PSI-F.

the thylakoid membranes, including the Lhca proteins, are present in more or less normal amounts [24,25]. The PSI-O protein is prevalent in stroma lamellae and only low amounts are detected in grana (Fig. 4), consistent with PSI-O being a subunit of PSI. Highly purified barley PSI with no contaminants detectable by SDS–PAGE [14] also contains the PSI-O protein (Fig. 4). In all the different preparations, the abundance of the PSI-O protein follows the abundance of the PSI-F protein (Fig. 4).

Most of the subunits of the PSI core complex in plants have homologues in cyanobacterial PSI. Exceptions are the subunits G, H and N that are only found in plants and green algae. PSI-M has not been found in angiosperms but is present in other plants and in cyanobacteria. A newly reported cyanobacterial subunit PSI-X [26] has not been found in eukaryotes. Searching of GenBank with the *Arabidopsis* PSI-O sequence showed the presence of a very large number of cDNA clones encoding similar proteins. Clearly, the PSI-O protein is present in higher plants, in mosses and in green algae. For example, cDNA clones from *Oryza sativa* (accession BF430472), *Marchantia polymorpha* (accession AU081654), and *Chlamydomonas reinhardtii* (accession AV634360), encode mature proteins with 84, 78 and 52% identity to the *Arabidopsis* PSI-O, respectively. However, no cyanobacterial sequences have any similarity with PSI-O. A wide comparison with all known proteins did not reveal any significant sequence similarities. Hence, the primary structure of PSI-O does not give clues to its specific function. Assuming that the central core of plant PSI is similar to the cyanobacterial PSI, it seems evident that PSI-O must be located at the periphery of the complex. With such a position, PSI-O would probably be in contact with one or more of the light-harvesting Chl *a*/*b*-binding proteins. These proteins are, like PSI-O, an addition to PSI that is specific to eukaryotes. Thus, PSI-O could have a role in interacting with peripheral light-harvesting proteins. The PSI-G and PSI-H proteins are eukaryote specific proteins, and both have a role in light-harvesting. PSI-H is required for proper interaction with LHCII during state transitions [7] and PSI-G is required for stabilising some of the LHCI subunits [27]. Future investigations should be directed at determining the function of PSI-O in plants and its location within the PSI complex.

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