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A previously found thylakoid membrane protein of 14 kDa (TMP14) is a novel subunit of plant photosystem I and is designated PSI-P

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Abstract We show that the thylakoid membrane phosphoprotein TMP14 is a novel subunit of plant photosystem I (PSI). Blue native/SDS–PAGE and sucrose gradient fractionation demonstrated the association of the protein exclusively with PSI. We designate the protein PSI-P. The presence of PSI-P subunit in *Arabidopsis* mutants lacking other PSI subunits was analyzed and suggested a location in the proximity of PSI-L, -H and -O subunits. The PSI-P protein was not differentially phosphorylated in state 1 and state 2.
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1. Introduction

Photosystem I (PSI) is a pigment–protein complex located in the photosynthetic membranes. It functions as an oxido-reductase, which accepts an electron on the luminal side delivered from PSII through a series of electron carriers and transfers it to the outer side of the membrane. There the electron is used to reduce $NADP^+$ which in turn will be used primarily for CO_2 assimilation. PSI is present in oxygen-evolving organisms such as cyanobacteria, algae and higher plants and its proper functioning is mediated by the intricate interplay of multiple subunits. Plant PSI is currently known to be composed of 14 core subunits (PSI-A to -L, PSI-N and PSI-O) and 4 light-harvesting complex I (LHCI) antenna proteins (Lhca1-4) [1] (Fig. 1). The function and putative location of several PSI subunits have been resolved by studying plants lacking products of different *PSI* genes [2]. Recently, analysis of crystals of pea PSI led to unraveling of the PSI structure at 4 Å resolution [3]. Light induces phosphorylation of several thylakoid membrane proteins [4,5]. The most abundant thylakoid phosphoproteins are LHCII (Lhcb1 and Lhcb2) and the PSII core proteins D1, D2 and CP43. Phosphorylation of LHCII proteins plays an important role in balancing the excitation energy between the two PSs in the process known as state transitions [6]. When the plastoquinone pool is oxidized, LHCII is not associated with PSI and this is known as state 1. When a change in light intensity or quality leads to reduction of the plastoquinone pool, LHCII becomes phosphorylated and a fraction moves to PSI (state 2).

Recently, *Arabidopsis* PSI-D was reported to be the first phosphorylated PSI subunit found [7]. Along with the discovery of PSI-D phosphorylation, a thylakoid membrane phosphoprotein of 14 kDa (TMP14, At2g46820) with unknown function was reported [7].

In the present paper, we demonstrate the exclusive localization of TMP14 in PSI, provide evidence that TMP14 is a novel subunit of this PS.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana (L.) Heyhn ecotype Columbia and *Nicotiana tabacum* cv. Petit Havanna wild-type (WT) plants were grown on compost in a controlled environment *Arabidopsis* chamber (Percival AR-60L, Boone, IA, USA) at a photosynthetic flux of 100–120 $\mu mol\ m^{-2}\ s^{-1}$ with an 8 h photoperiod and 70% relative humidity. Seedlings of WT barley (*Hordeum vulgare* cv. Svalöfs Bonus) and the *viridis-zb*[63] mutant were grown in vermiculite for 6 days at 21 °C in constant white light at a photon flux of 75 $\mu mol\ m^{-2}\ s^{-1}$. The *Arabidopsis* mutants and transformants deficient in individual PSI subunits have been described before (see [2] for a review).

2.2. Thylakoid preparation and isolation of PSI complexes

Thylakoids were prepared from 6- to 8-week-old *Arabidopsis* leaves as described earlier [8] except that 10 mM NaF was added to all buffers to inhibit phosphatase activity. Thylakoids were stored at −80 °C. Total chlorophyll (Chl) was determined in 80% acetone [9]. PSI complexes were isolated by solubilization of the thylakoid membranes (1 mg Chl/ml) for 10 min at 0 °C with 1% dodecyl-β-maltoside (β-DM; Sigma, St. Louis, MO, USA) in the presence of NaF, followed by sucrose gradient ultracentrifugation as previously described [11]. For analysis of phosphorylation of PSI and LHCII in state 1 and state 2 the plants were illuminated with orange (state 2) or red (state 1) light

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Abbreviations: PS, photosystem; LHC, light-harvesting complex; WT, wild-type; Chl, chlorophyll; β-DM, dodecyl-β-maltoside; BN, blue native

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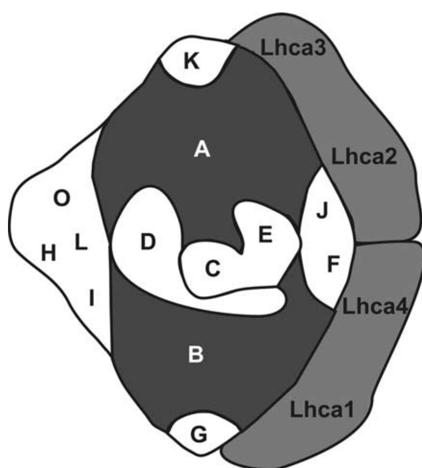

Fig. 1. Schematic model of PSI viewed from the stromal side. The PSI-N subunit is on the luminal side and not visible in this view.

and samples were prepared using digitonin and sucrose gradient centrifugation as previously described [12]. For blue native (BN)–PAGE *Arabidopsis* thylakoid-membrane subfractions were isolated using digitonin as previously described [13].

2.3. SDS–PAGE and immunoblotting

TMP14 antibody was raised by immunizing a rabbit with a synthetic peptide (VKTAQEAWEKVDDK), conjugated to keyhole limpet hemocyanin (Innovagen, Lund, Sweden). Phospho-threonine polyclonal antibody was from Cell Signaling (Beverly, MA, USA). Thylakoid and PSI proteins were separated using 12% Criterion SDS-gels (Bio-Rad, Hercules, CA, USA). Immunoblotting was carried out by transferring separated proteins to a PVDF membrane (Bio-Rad) using a semi-dry electroblotter. The membrane was incubated with polyclonal antibodies as indicated in the figure legends. Signal was detected with a chemiluminescent detection system (SuperSignal, Pierce, IL, USA) after incubation with horse radish peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark) and recorded using a cooled CCD camera.

2.4. BN–PAGE

BN–PAGE was performed essentially as described [14]. The samples solubilized with β-DM were electrophoresed in the first dimension on 5–12.5% acrylamide gradient gels (Hoefer Mighty Small, Amersham Pharmacia Biotech, Uppsala, Sweden). After electrophoresis the lanes were cut out, incubated in sample buffer [15] containing 5% β-mercaptoethanol, and run in the second dimension by SDS–PAGE on 15% gels with 6 M urea.

2.5. Isoelectrofocusing

Stroma lamellae (150 μg protein) were solubilized in 320 μl rehydration buffer (2 M thiourea, 8 M urea, 4% CHAPS, 20 mM Tris–base, 0.5% IPG buffer, pH 3–10, 100 mM DTT, and a trace of bromphenol blue) for 3 h at room temperature and centrifuged at 13000 g for 20 min to remove insoluble material. ReadyStrip™ IPG strips, pH 3–10 (Bio-Rad) were rehydrated for 12 h in the sample suspension at 20 °C. Isoelectrofocusing in the first dimension using the Protean IEF apparatus (Bio-Rad) and SDS–PAGE on 14% gels in the second dimension were performed according to manufacturer's instructions. After electrophoresis, the proteins were visualized by immunoblotting or by silver staining.

2.6. Electrospray ionization MS

Protein spots were digested in-gel with trypsin (Sequencing Grade Modified, Promega, Madison, WI, USA) according to Shevchenko et al. [16]. Peptides were desalted on a C_{18} reversed-phase ZipTip (Millipore, Billerica, MA, USA) and eluted with 50% acetonitrile, 1% formic acid. Electrospray ionization tandem MS was performed using API Q-STAR *Pulsar* (Applied Biosystems, Foster City, CA, USA) equipped with a nano-electrospray ion source (MDS Protana, Odense, Denmark) with instrument settings recommended by Applied Biosystems.

3. Results

3.1. TMP14 is associated with PSI

In order to study the localization of TMP14, *Arabidopsis* thylakoid membranes were fractionated into stroma lamellae and grana. The samples were subjected to BN–PAGE to separate the protein complexes followed by separation of the protein subunits of the complexes in a second dimension by SDS–PAGE. Immunoblotting revealed that TMP14 in the stroma lamellae co-migrated exclusively with the PSI complex (Fig. 2). In grana the TMP14 protein was not detected (data not shown).

To independently confirm the association of TMP14 with PSI, *Arabidopsis* thylakoids were solubilized with β-DM and applied on a sucrose gradient to fractionate PSI, PSII and LHCII. Immunoblotting using TMP14 antibody showed a clear enrichment of the PSI fraction with TMP14 (Fig. 3A). To see whether TMP14 was associated with other thylakoid membrane complexes the entire sucrose gradient was harvested in 25 fractions and analyzed by SDS–PAGE. One gel was stained while another was subjected to immunoblotting. The result showed that the presence of TMP14 was restricted to fractions containing PSI (Fig. 3B). Antibody against PSI-G (a PSI subunit known to be located at the periphery of the PSI complex) was used as a control to localize the PSI fraction. This antibody clearly shows the concentration of PSI in fractions 1–8. A small amount of PSI-G was found in fractions of the gradient where free LHCII is found. The latter has been observed earlier and is considered to be due to partial release

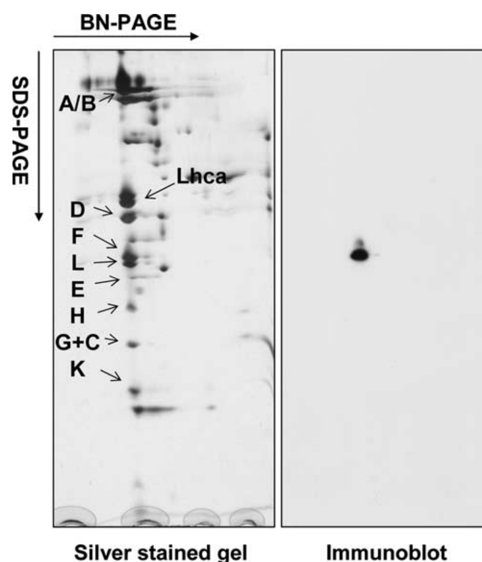

Fig. 2. Separation of the stroma thylakoid proteins by 2D BN–PAGE demonstrates TMP14 association with PSI. Immunoblotting with TMP14 antibody shows that TMP14 migrated together with PSI under non-denaturing conditions. After denaturation TMP14 was found exclusively among the PSI subunits at the same position as PSI-L. The letters designate the individual PSI subunits [10].

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