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Evidences for interaction of PsbS with photosynthetic complexes in maize thylakoids

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Abstract

The PsbS subunit of Photosystem II (PSII) has received much attention in the past few years, given its crucial role in photoprotection of higher plants. The exact location of this small subunit in thylakoids is also debated. In this work possible interaction partners of PsbS have been identified by immunoaffinity and immunoprecipitation, performed with mildly solubilized whole thylakoid membrane. The interacting proteins, as identified by mass spectrometry analysis of the immunoaffinity eluate, include CP29, some LHCII components, but also components of Photosystem I, of the cytochrome b_6f complex as well as of ATP synthase. These proteins can be co-immunoprecipitated by using highly specific anti-PsbS antibodies and, vice-versa, PsbS is co-immunoprecipitated by antisera against components of the interacting complexes. We also find that PsbS co-migrates with bands containing PSII, ATP synthase and cytochrome b_6f as well as with LHCII-containing bands on non-denaturing Deriphat PAGE. These results suggest multiple location of PsbS in the thylakoid membrane and point to an unexpected lateral mobility of this PSII subunit. As revealed by immunogold labelling with antibody against PsbS, the protein is associated either with granal membranes or prevalently with stroma lamellae in low or high-intensity light-treated intact leaves, respectively. This finding is consistent with the capability of PsbS to interact with complexes located in stroma lamellae, even though the exact physiological condition(s) under which these interactions may take place remain to be clarified.

Keywords: PsbS; Photosynthetic complex; Maize thylakoid

1. Introduction

PsbS is a small subunit of Photosystem II (PSII), isolated for the first time by Ljunberg et al. [1] and cloned by Herrmann et al. [2]. This 22 kDa protein, belonging to the Cab superfamily [3], received renewed attention when its crucial role in non-photochemical quenching (NPQ) was established by the Niyogi group, who functionally characterized a PsbS-less *npq4* mutant of *Arabidopsis thaliana* [4]. In general, over-excitation of PSII is prevented by a number of mechanisms characterized by different response time [5]. A series of processes involved in fast photoprotection are collectively named NPQ and are monitored by the quenching of Chla fluorescence (e.g. [6–8]). Under most physiological conditions, the major component of NPQ is qE, which dissipates excess absorbed energy as heat. qE depends on "energizing" of the thylakoid membrane and is activated by lumen acidification. The key players responsible for qE have been identified during the past decade and are the PsbS protein [4,9], pigments of the xanthophyll cycle [10], especially zeaxanthin, and components of the light-harvesting apparatus of Photosystem II (LHCII) [11–13].

PsbS seems to have more than one location in the PSII supercomplex: it has been proposed that it is located in the region of interaction between PSII core (Photosystem II comprising inner antenna proteins CP43 and CP47 but not Lhcb) and CP29 [14,15]. On the other hand, direct interaction of PsbS with CP29 has not yet been detected [15]. Alternatively, PsbS may be associated more closely with either PSII core or Lhcb proteins, depending on lumen pH [16]. Furthermore,

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Nield et al. [17] concluded that PsbS is not contained within the LHCII–PSII supercomplex and reported that solubilization of PSII particles (BBY membranes) by 25 mM β-dodecyl-maltoside (β-DM) was sufficient to induce migration of PsbS in sucrose gradient independently of PSII. As a matter of fact, PsbS has never been identified within the LHCII–PSII supercomplex by structural analysis [18].

Elucidation of interaction partners of PsbS would improve our understanding of its location within the membrane. Three different protocols have been reported for purification of PsbS. One of these used 0.5% sodium cholate for solubilization [19], obtaining pure PsbS, capable of binding zeaxanthin in a reconstituted system. In another work, PsbS was purified from PSII preparations, solubilized with 0.3% β-DM and subjected to cation exchange chromatography [15]. The third approach to isolate PsbS applied 0.5% β-DM to G&Y PSII particles [3]. Importantly, in all three cases, purification was performed starting either from isolated Photosystem II cores or PSII-enriched BBY membranes by relatively strong solubilization procedures. Furthermore, isolation of BBY or PSII itself requires solubilization of thylakoids by a non-ionic detergent, such as Triton X100. These approaches, while allowing isolation of pure PsbS, are not expected to maintain association with the interaction partner proteins, which are probably held in place by weak hydrophobic and/or electrostatic forces.

In this work we undertook a different approach, exploiting a highly-specific anti-PsbS antibody. The antibody, obtained against recombinant PsbS, recognizes the stroma exposed loop between the second and third transmembrane helices of PsbS, and can detect both monomeric and dimeric forms of this subunit [16]. Immunopurification of PsbS was performed using mildly solubilized whole thylakoids. This allowed us to identify the possible interaction partners of PsbS and the effective interaction was further demonstrated by pull-down assay, a widely-accepted method to prove protein–protein interaction (see e.g. [20,21]). In this context it is worth mentioning that an important result has been achieved in the field of mitochondrial ion channels, using co-immunoprecipitation on solubilized inner mitochondrial membrane preparation. The authors showed that at least four mitochondrial membrane proteins interact with succinate dehydrogenase, as part of a multiprotein complex which confers mitochondrial ATP-sensitive potassium channel activity [22]. In thylakoids, the membrane protein Alb3 has been shown to associate with cpSecY translocase by co-immunoprecipitation experiments [23]. In the present paper we provide evidence for a multiple location of PsbS in the thylakoid membrane and for its capability to interact with various protein complexes that are involved in photosynthesis.

2. Experimental procedures

2.1. Plant material and growth conditions

Kernels of maize (*Zea mays*) were soaked in tap water overnight and planted in vermiculite. Plants were grown for 14–20 days at 23 °C, with a light intensity of 50 μE m^{-2} s^{-1} and 12 h photoperiod. Relative humidity was 70%.

2.2. Isolation of thylakoids

Plants were homogenized with a blender and ground in grinding buffer (50 mM Tricine pH 7.8, 5 mM $MgCl_2$, 10 mM NaCl and 0.33 M sorbitol). After filtering through cotton gauze, thylakoids were pelleted by centrifugation for 3 min at 10,700×*g* at 4 °C. For separation of mesophyll chloroplasts, the slurry was filtered through two layers of 30 μm nylon mesh and centrifuged for 10 min at 1500×*g* [24]. Pellets were resuspended in grinding buffer without sorbitol. Thylakoids were again pelleted for 10 min in the same conditions as above and then resuspended in 100 mM sorbitol, 50 mM Tricine pH 7.8, 10 mM NaCl and 5 mM $MgCl_2$ (P3).

2.3. Immunoaffinity

PsbS protein was purified by immunoaffinity chromatography in batch as described by Harlow-Lane with minor modifications [25]. The immunoaffinity resin was prepared as follows: 500 μl of anti-PsbS serum was incubated with 1 ml of protein A Sepharose beads (Amersham Biosciences) for 2.5 h at room temperature. The resin was washed twice with 10 volumes of 0.2 M sodium borate (pH 9.0). IgG molecules were cross-linked to protein A by adding 50 mM dimethylpimelimidate (Sigma) and incubating the resin for 3 h. The reaction was stopped by a series of washes with 0.2 M ethanolamine (pH 8.0) and PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4). The formation of covalent bond between the anti-PsbS antibody and protein A was checked by SDS-PAGE: as expected, no 55 kDa protein, corresponding to the heavy chains of IgG, was visible on the gel (not shown). Thylakoid membranes were solubilized (500 μg/ml chlorophyll) with 0.3% (wt/vol) β-DM for 30 min on ice and centrifuged for 3 min at 13,000×*g*. The IgG-coupled resin was equilibrated with 50 mM Tris/HCl (pH 7.5) and then incubated overnight at 4 °C with the supernatant containing β-DM-solubilized proteins in the presence of protease inhibitor mixture (Sigma). Solubilized thylakoids were added in excess with respect to beads (10:1 v/v ratio). Overnight incubation [23] of solubilized thylakoids with the activated beads was performed in the presence of a cocktail of protease inhibitors (in order to avoid proteolytic degradation). No proteins bound to ProteinA beads without anti-PsbS linked to it. Unspecifically bound proteins were eluted by repeated washing with 0.1% Tween20 in PBS and then with 250 mM NaCl in 50 mM Tris/HCl (pH 7.5). PsbS protein was eluted with three volumes (equal to the resin one) of 50 mM Tris/HCl (pH 7.5), 2 M NaCl and 0.03% β-DM. Immunoaffinity performed in more stringent conditions [22] (thylakoid solubilization with Triton X-100 and presence of 10% ethylene glycol as well as of 100 mM KCl during incubation with beads) did not allow significant binding of PsbS to the immobilized antibody (not shown).

2.4. Immunoprecipitation

Solubilized thylakoids (at 500 μg/ml chlorophyll concentration with 0.3% β-DM for 30 min on ice) were centrifuged and 2 ml of supernatant was gently shaken with 50 μl of serum overnight at 4 °C in the presence of protease inhibitors. The mixture was then incubated with 50 μl of Protein A Sepharose beads for 40 min, centrifuged for 30 s (13,000×*g*), and washed three times with PBS. Beads, boiled for 5 min with 4% SDS, 20% glycerol, 125 mM Tris, pH 6.8, 10% β-mercaptoethanol, were loaded on Urea/SDS-PAGE. These assays gave the same results when immunoprecipitation was performed for 2 h instead of overnight (not shown).

2.5. Urea/SDS-PAGE and immunoblotting

Gel electrophoresis and Western blot were performed as described previously [26]. Samples were solubilized with a buffer (SB) containing 3% SDS, 33 mM DTT, 10% glycerol, 42 mM Tris, pH 6.8, except when otherwise specified. Anti-PsaF and anti-Lhca2 and anti-Lhca3 antibodies were kind gifts from Professors Jensen and Bassi and were used at 1: 5000 and 1:1000 dilutions, respectively. Anti-PsbS, anti-LHCII, anti-CP29 and anti-cytochrome f were used at 1: 10,000, 1: 1000, 1: 2000 and 1:5000, respectively. Anti- PsaD, anti-Lhca4 and antibody against the β-subunit of ATP synthase were from Agrisera (http://www.agrisera.se). Horse-radish peroxidase coupled anti-rabbit IgG was used as a secondary antibody and blots were developed using the ECL system.

2.6. Deriphat PAGE and immunoblotting

Deriphat PAGE was performed according to Poggese et al. [27]. Thylakoids were solubilized with 2% β-DM for 15 min. For 2D-PAGE, the band containing thylakoids separated on Deriphat was soaked for 1 h with SB and then loaded on an urea/SDS-PAGE.

2.7. Mass spectrometry

Protein digestion was performed according to Shevchenko et al. [28], with minor modifications. The dried tryptic digest samples were reconstituted in 10 μl of 0.5% TFA in water and were purified with a Zip-TipC18 (Millipore). For electrospray MS analysis, the peptides were eluted in 50% acetonitrile containing 0.2% formic acid. Data were collected on a Micromass Q-Tof spectrometer (Manchester, UK) (capillary voltage: 3000–3200 V; cone voltage: 45 V; scan time: 1 s; interscan: 0.1 s). Spectra were analyzed using Micromass Mass-Lynx. The MASCOT program (www.matrix-science.com) was used to search for all MS/MS spectra against the Swiss-Prot database. The parameters were set to give parent ion mass tolerance of 1 Da and fragment mass tolerance of 0.8 Da, and up to two missed Trypsin.

2.8. Immunogold labelling

For the immuno-electron microscopy the tissue samples were fixed at room temperature for 2 h in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) (PBS), dehydrated in ethanol and embedded in London Resin White. Ultrathin sections were cut with an Ultramicrotome (Ultracut, Reichert-Jung), picked up on gold grids, washed with PBS, incubated for 20 min. on 1% bovine serum albumin in PBS and treated with the anti-PsbS antibody diluted 1:1000. After washing with PBS the sections were incubated with colloidal gold (10 nm) conjugated with goat anti-rabbit IgG (Sigma-Aldrich, Italy). Sections were then stained with uranyl acetate followed by lead citrate and examined with the electron microscope (TEM 300, Hitachi) operating at 75 kV. Control experiments were performed by eliminating the incubation of sections with the primary antibody (not shown).

3. Results

Purification of PsbS was done by immunoaffinity using thylakoid membranes of maize from mesophyll chloroplasts. The PsbS antibody used was highly specific for PsbS and did not cross-react with other plastidial proteins in Western blot assay. Thylakoid membranes, isolated in the presence of 5 mM $MgCl_2$ (in order to maintain membrane stacking) were gently solubilized for 30 min with 0.3% β-DM and incubated with Sepharose-ProteinA beads to which the anti-PsbS antibody had previously been covalently linked [25]. This procedure resulted in the binding of the monomeric form of PsbS to the beads (Fig. 1A, left lane), while both monomeric and dimeric forms were still present in the fraction which did not bind to the beads as revealed by SDS-PAGE (Fig. 1A, right lane). We believe that in the dimeric form in the mildly solubilized sample, the epitope of PsbS is not accessible, resulting in lack of binding to the affinity column. Monomeric PsbS was eluted by a relatively mild treatment, with 2 M NaCl [29].

While Western blot of the eluate with anti-PsbS antibody detected a single band at the expected molecular weight of PsbS (22 kDa) (Fig. 1B), silver-staining revealed the presence of several proteins (Fig. 1C). Given the lack of recognition by anti-PsbS of the other proteins in the eluate, we considered them as possible interaction partners of PsbS. Proteins of the eluate were separated on SDS-PAGE, excised and analyzed by tandem MS.

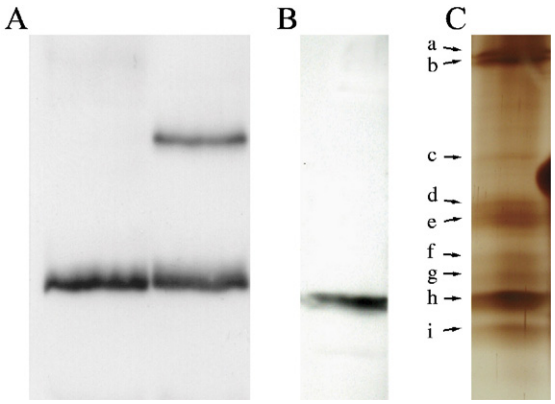

Fig. 1. Immunoaffinity purification of PsbS identifies its possible interaction partners. (A) Right lane: both monomeric and dimeric forms of PsbS are present in solubilized thylakoids recovered after overnight incubation with anti-PsbS containing bead (50 μl of recovered thylakoids loaded). Left lane: 10 μl Protein-A beads, containing anti-PsbS antibody, after overnight incubation with solubilized thylakoids. The monomeric form of PsbS binds to the beads. Beads were loaded following solubilization with sampling buffer (see Experimental procedures). The eluate contained PsbS (B) as shown by Western blot. (C) Silver-stained SDS-PAGE pattern of the eluate obtained with 2 M NaCl. 1.5 ml of the eluate was treated with 4 volumes of ice-cold acetone, centrifuged and resuspended in standard SB. Aliquots were loaded in B and C. Bands marked with letters were cut and subjected to mass spectrometry analysis. Please note that not all bands were intensive enough for MS analysis (see e.g. two bands between b and c). The result of MS/MS analysis is shown in Table 1. In band f no thylakoid protein could be identified with high score.

The analysis results, reported in Table 1, as expected, revealed that the most prominent band of the eluted material corresponds to PsbS protein (band h of Fig. 1C). The MS/MS spectrum is reported for PsbS in Fig. 2. However, α, β and γ subunits of ATP synthase, CP29, LHCII and LHC I components and cytochrome b_6 of the cytochrome b_6f complex were also identified. Furthermore, two small subunits of Photosystem I (PSI), namely PSI-F and PSI-L, were unexpectedly also found by mass spectrometry in the eluate.

To further prove the actual PsbS interaction partnership with the proteins found in the eluate of immunoaffinity purification, we performed immunoprecipitation experiments. Fig. 3 shows co-immunoprecipitation of LHCII, CP29 and PsaF (Fig. 3A). The same figure also shows that anti-CP29 and anti-PsaF antibodies can pull-down PsbS when used to immunoprecipitate their own antigens. These assays gave the same results when immunoprecipitation was performed for 30 min or 2 h. It is worth pointing out that anti-PsaF and anti-CP29 are also highly-specific antibodies that show no cross-reaction with PsbS in Western blot (not shown). Anti-LHCII antibodies were not used for this reciprocal pull-down test because they cross-react with PsbS when used at the relatively high concentrations needed for pull-down assays (not shown). In Fig. 3B the results of co-immunoprecipitation after stronger solubilization of the thylakoid membrane (0.5% deoxycholate) are shown. It clearly appears that the interaction with LHCII and CP29 was significantly disrupted by this detergent. In contrast, PSI-F was still present in the immunoprecipitate, indicating a relatively stronger interaction with PsbS.

Table 1
Proteins in the immunoaffinity eluate as identified by MS/MS analysis

Band on gel	Accession number (NCBI database)	Name of protein	Predicted MW (kDa)	Identified peptide sequences
a	gi 902219	α subunit of ATP synthase	55,7	KTAVATDTILNQKG
b	gi 552732	β subunit of ATP synthase	54	RFVQAGSEVSALLGRM
c	gi 50937699*	γ subunit of ATP synthase	39,7	KVALVVLTGERG
d	gi 2326947	CP29	31,4	KNEAGGIIGTRF
e	gi 22230	LHCII Type I	27,9	KAKPAAASGSPWYGPDRV
	gi 22355	LHCII Cab-M9	28	RELEVIHSRW
	gi 22224	LHCII Type I	27,8	KVAASGSPWYGPDRV
	gi 452341	LHCII Type II	24,7	VGGGPLGEGLDK
g	gi 30692874*	Lhca4	27,7	KNPGSVNQDPIFK
h	gi 33867383	PsbS	27,7	RGALGLSEGGPLFGFTK
i	gi 5734518*	PsaF	24,1	RGFIWPVAAYRE
	gi 77554828*	PsaL	22	RTAVSPLLRG
	gi 902251	Cytochrome b_6	26,2	KIVTGVPEAIPVIGSPL

For each protein several peptides were identified (from 2 to 18)—one representative peptide sequence with the highest score is reported for simplicity. Accession numbers refer to NCBI database. For some proteins (marked with *) a maize homolog could not be found in the database—accession numbers refer to *Oryza* or *Arabidopsis* proteins, containing the identified sequences. Theoretical MW of the preproteins are shown.

The question arises as to whether PsbS interacts with single proteins or with complexes. For this reason we checked whether other components than those identified by MS analysis of PSI and cytochrome b_6f complexes can be found in the immunoaffinity eluate by Western blot. PsaD, Lhca2, Lhca3 of Photosystem I supercomplex as well as cytochrome f of the latter complex were indeed present in the eluate (Fig. 4A). We would like to note that MS analysis of the eluate was performed on acetone-precipitated sample in order to have sufficient quantities of the proteins in silver-stained bands for MS analysis, while Western blot was performed on the eluate without any treatment. The various components that are present in the eluate according to Western blot, but were not found by MS analysis, may either be lost during precipitation with acetone (as revealed by silver staining of non-treated eluate, not shown) and/or may not be present in sufficient quantity for MS assay. In any case, given that the anti-cytochrome f antibody was able to pull down PsbS and vice versa (Fig. 4B) and cytochrome b_6 was found in the eluate, it is plausible to propose that PsbS interacts with the whole cytochrome b_6f complex. The above results are in accordance with previous reports showing that the mild solubilization conditions used here are not sufficiently strong to dismantle PSI and the cytochrome b_6f complexes (see e.g. [30]). Similar considerations can be taken for the ATP synthase complex (Fig. 4B). Antibodies against the β subunit of ATP synthase pulled down PsbS efficiently. Given the hydrophilic nature of this subunit, as well as the fact that we identified three distinct subunits of ATP synthase in the eluate, it is quite probable that PsbS does not interact directly only with the β subunit, but rather with the whole complex. Also in the case of this complex, mild solubilization conditions leave at least AtpA, B and C together, as seen in BN-PAGE (e.g. [31]). To further

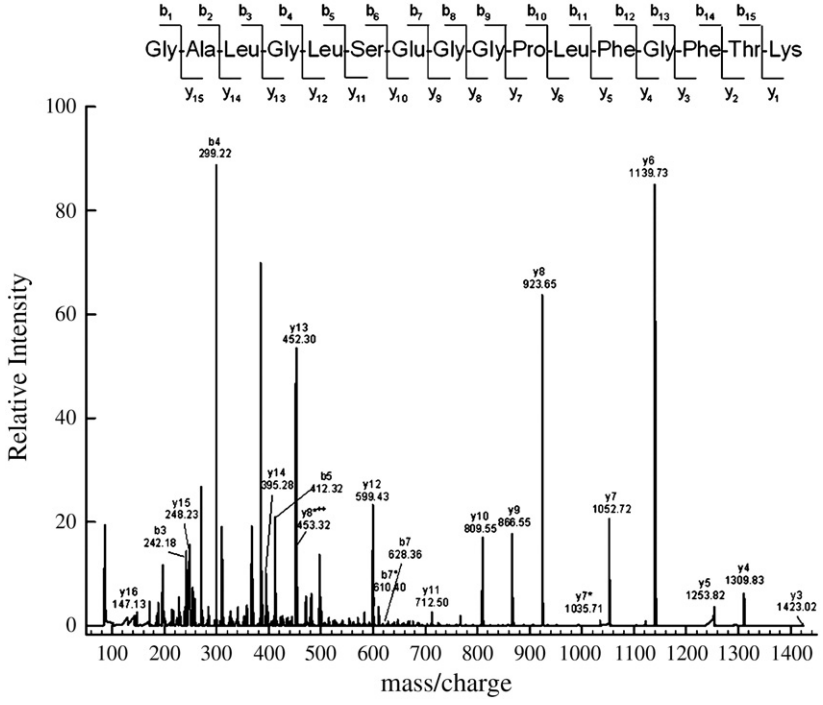

Fig. 2. Mass spectrometry analysis confirms the presence of PsbS in the fraction eluted during immunoaffinity purification. MS/MS spectrum of double charged precursor ion with *m/z* 775,9 detected in ESI-MS spectrum of tryptic digest of band h from the gel shown in Fig. 1C. One of the peptides is indicated in the spectrum together with location of y and b ions.

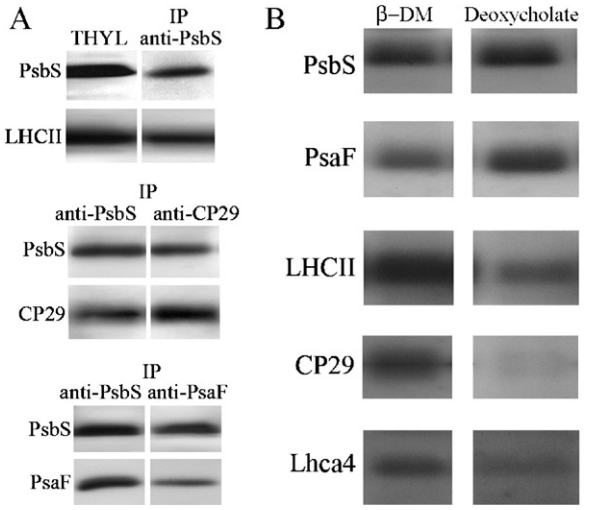

Fig. 3. Pull-down assays confirm interaction between PsbS and CP29, LHCII, Lhca4 and the F subunit of PSI. (A) Upper panel: LHCII can be immunoprecipitated by anti-PsbS antibody. Left lanes: Control maize thylakoids (containing 5 μg chlorophyll) blotted with anti-PsbS (upper panel) or anti-LHCII (lower panel) antibodies. Right lanes: immunoprecipitates pulled down with anti-PsbS, as described in Experimental procedures. In the upper panel 10 μl beads were loaded and anti-PsbS antibody was used while in the lower panel 30 μl beads were loaded and Western blot was performed with anti-LHCII antibody. Middle panel: anti-PsbS antibody pulls down CP29 (left part) and vice versa, anti-CP29 antibody immunoprecipitates PsbS (right part) from solubilized whole thylakoids. 50 μl beads/lane are loaded. Upper part: blotted with anti-PsbS antibody; lower part: blotted with anti-CP29 antibody. Lower panel: anti-PsaF antibody pulls down PsbS and vice versa. Immunoprecipitates obtained by using anti-PsbS (left part) or anti-PsaF (right part), blotted with anti-PsbS (upper panel) or anti-PsaF (lower panel) antibodies are shown. 50 μl beads/lane were loaded following solubilization of beads with SB containing β-mercaptoethanol. (B) Immunoprecipitates were obtained using anti-PsbS antibody on whole thylakoids (0.5 mg/ml chlorophyll) solubilized either with 0.3% β-DM (left lanes), or with 0.5% (w/v) sodium deoxycholate (right lanes) for 30 min. on ice. Pull down assays in these two conditions were performed in parallel and samples were loaded on the same SDS-PAGE and blotted with various antibodies to detect the indicated proteins. Comparison of right and left lanes reveals that e.g. interaction between CP29 and PsbS was largely disrupted in the presence of deoxycholate.

investigate whether in the immunoaffinity purification proteins in the eluate are present as single components or as whole complexes, the eluate was loaded on non-denaturing Deriphat PAGE. As observable in Fig. 5A, two bands at high MWs were revealed. Although we do not know the exact protein composition of these bands, both contain the D subunit of PSI (Fig. 5A) and migrate with an apparent weight close to what we observe with PSI complex in Deriphat-PAGE (Fig. 5A), indicating that at least in the case of PSI, the subunits we identified in the eluate, are part of a complex.

If solubilization of the thylakoid membrane was incomplete, a priori, immunoprecipitation could bring down a number of proteins that are not specifically interacting with each other but just present in the same partially solubilized membrane fraction. However, despite the co-existence of ATP synthase and cytochrome b_6f complex in the same region, i.e. in stroma lamellae, antibodies against the β subunit of ATP synthase pulled down PsbS but not cytochrome f. Similarly, anti-PsaD (PSI subunit) and anti-cytochrome b_{559} (PSII subunit) anti-

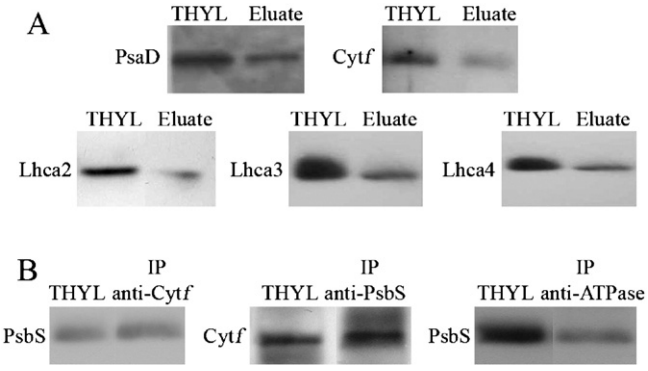

Fig. 4. Immunoaffinity eluate contains various components of photosynthetic complexes. (A) Thylakoids (2 μg chlorophyll/lane) as well as 50 μl of the eluate were loaded on SDS-PAGE and subsequently blotted with the indicated antibodies. (B) Immunoprecipitations have been performed by using the indicated antibodies (anti-Cytf, anti-PsbS and anti-ATP-ase). Thylakoids and IP samples were assayed as shown on figure. The presence of ATP-ase in the immunoprecipitate obtained by using anti-PsbS antibody could not be revealed because of an overlapping migration of the heavy chain of IgG with β subunit of ATP synthase.

bodies immunoprecipitated PsbS, but not cytochrome f (see Fig. 5B). Furthermore, both stromal and granal membranes were solubilized in our conditions, as indicated by the presence of various PSII proteins (cytochrome b_{559}, psbS and D2) in the supernatant obtained following solubilization. In light of these results, we conclude that pull down assays with anti-PsbS very

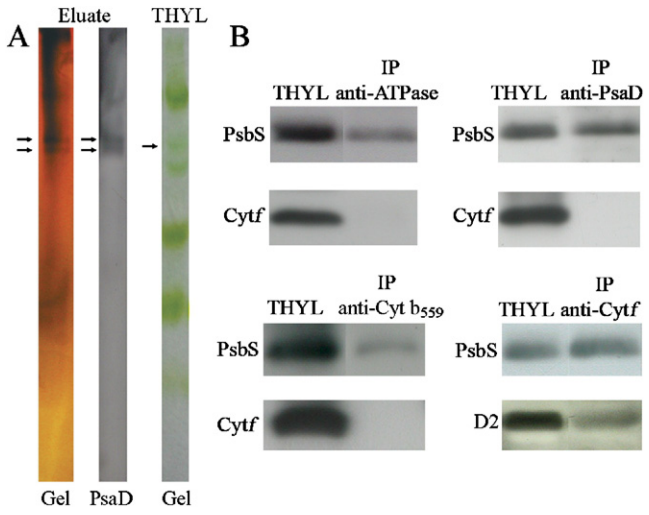

Fig. 5. PsbS interacts with complexes in a specific way. (A) Immunoaffinity eluate was loaded on non-denaturing Deriphat-PAGE and stained with silver (left lane) or blotted with anti-PsaD antibody (middle lane). Migration of both bands were similar to that obtained for PSI complex when loading whole thylakoids (right lane). All three lanes shown are from the same gel. (B) Immunoprecipitations were performed as described for all other figures. The results shown here confirm that interaction of various complexes is not unspecific under our solubilization conditions, given that the used antibodies pull down PsbS, but not cytochrome f (anti-cytf). Please note that PsbS is efficiently pulled down by anti-cytf antibody, while only small amount of D2 is found in the immunoprecipitate, indicating that at least a part of PsbS is not bound to PSII core. In all cases, thylakoid and IP samples shown were first blotted with anti-PsbS and then reblotted with anti-cytf antibodies, or vice versa, in order to avoid any difference in signal due to unequal loading.

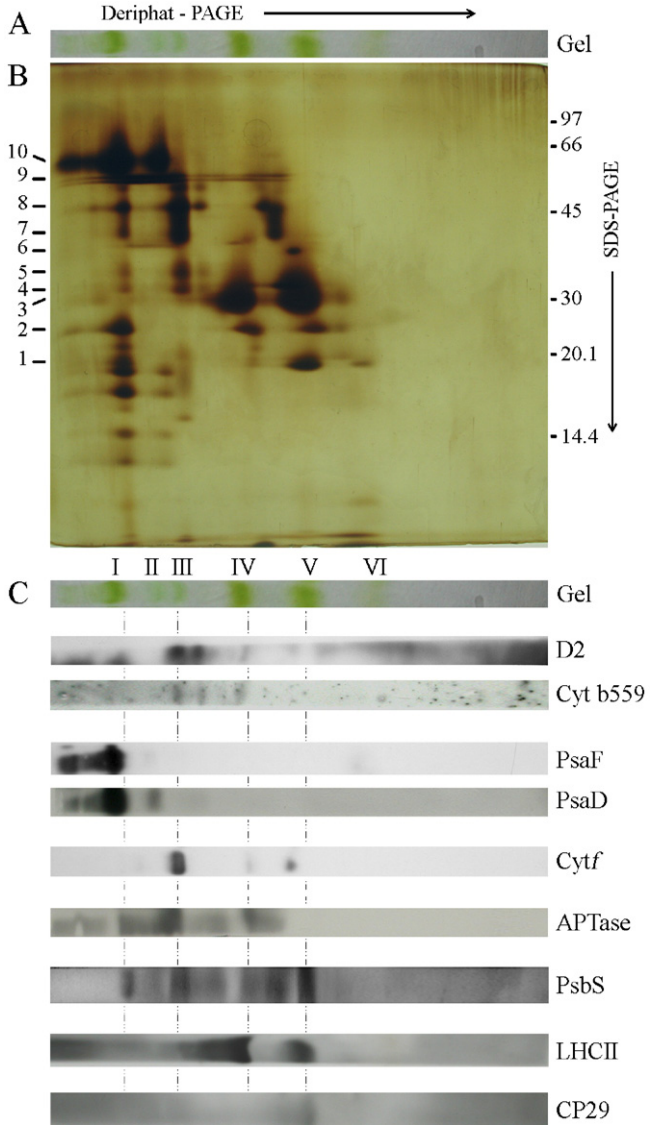

Fig. 6. PsbS co-migrates with various photosynthetic complexes in Deriphat-PAGE. Solubilized thylakoids were loaded on Deriphat PAGE (A) and the gel so obtained was loaded on SDS-PAGE in order to separate all protein components of the different bands (B). Proteins were visualized by silver-staining. Numbers correspond to: 1: PsbS; 2: LHCI type I; 3: LHCII; 4: D1; 5: D2; 6: cytochrome f and γ subunit of ATP synthase; 7: CP43; 8: CP47; 9: α and β chains of ATP synthase; 10: PsaA and PsaB. These spots were identified on the basis of MS analysis. The lanes obtained on Deriphat-PAGE were also transblotted and decorated with the indicated antibodies (C). Dashed lines show the position of PsbS where migration of this protein coincides with the position of complexes. Green bands contain the following complexes: (I) PSI, PSII core, ATP synthase; (II) PSI core, ATP synthase; (III) PSII core, ATP synthase, Cyt b_6f; (IV) LHCII trimers; (V) LHCII monomers; (VI) free pigments.

likely indicate specific interactions. The exact conditions under which these partnerships occur, has still to be established, by using other methodologies, since pull-down assays cannot be carried out on non-solubilized membranes that are kept under e.g. specific illumination conditions during the whole procedure.

PsbS is considered up to now as a bona fide PSII subunit (but see Introduction). The results reported above indicate however, that PsbS may be associated with different complexes in the plane of the thylakoid membrane. To address this point, we established whether PsbS co-migrates exclusively with PSII core in non-denaturing Deriphat-PAGE. This method is generally used to separate PSI, monomeric PSII core, LHCIIb and minor antenna proteins [32]. Fig. 6 shows the presence of distinct coloured bands (A) as well as the protein composition of each band obtained in 2D gel (Deriphat-PAGE in first dimension and SDS-PAGE in second dimension) (Fig. 6B). Western blots of Fig. 6C indicate the position of the migration of different complexes in Deriphat-PAGE, based on the use of specific antibodies for each complex. PsbS co-migrates with the upper band that contains prevalently PSI but also some PSII proteins (in accordance to results of silver-stained 2D-PAGE), with PSII core, cytochrome b_6f complex and with LHCII-containing bands. Since PsbS alone has only 22 kDa apparent molecular weight, a co-migration with the above complexes means that PsbS is relatively strongly associated with them. Whether PsbS is found together with ATP synthase or not cannot be determined since PSII core and ATP synthase migrate in close vicinity, in accordance to results obtained also by BN-PAGE [31].

The above data strongly indicate a lateral mobility of PsbS within the thylakoid membrane. To prove mobility of PsbS also in a physiological, intact system, the location of PsbS was

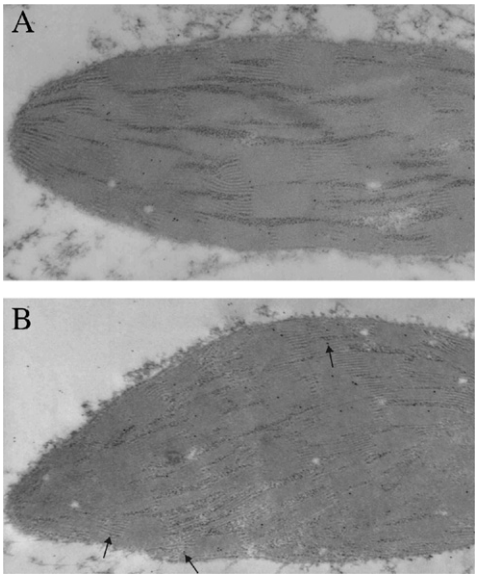

Fig. 7. Lateral mobility of PsbS as revealed by immunogold labelling. Shown are representative images of mesophyll chloroplasts from intact leaves, illuminated with (A) low intensity light (50 μE m^{-2} s^{-1}) or (B) high intensity light (350 μE m^{-2} s^{-1}) for 30 min. While in (A) PsbS can be visualized in the grana region, in (B) the gold particles showing the presence of PsbS are observable in the stroma lamellae as well (see e.g. arrows). Immunogold labelling procedure does not permit to obtain an image that is normally seen by transmission electron microscopy. Membranes can be clearly recognized only in the non-stacked, stroma lamellae regions. The experiment was repeated giving similar result and showed that in the low-light illuminated sample 70% of the gold particles were located in the grana region, while in high-light illuminated leaves 36%, 26% and 38% of gold particles were found in grana, margin and stroma lamellae regions, respectively.

investigated using immunogold labelling in intact maize leaves, especially in mesophyll chloroplasts, known to display stacked grana and stroma lamellae. PsbS is located in stacked grana membranes in the chloroplasts of leaves that were illuminated with relatively low-intensity light (50 μE m^{-2} s^{-1}) (Fig. 7A), in accordance with PsbS being a PSII subunit. As mentioned above, the exact conditions in which interactions with stroma lamellae-located complexes may take place have to be determined. However, given that PsbS is crucial for photoprotection, we examined its location in leaves that were illuminated with high-intensity light (350 μE m^{-2} s^{-1}), sufficient to induce non-photochemical quenching [16]. In this condition, a significant part of PsbS can be revealed in the stroma lamellae (see arrows) and in the grana margin region (Fig. 7B).

4. Discussion

In this work we applied immunopurification to isolate PsbS protein and identify its interaction partners directly from thylakoid membranes. Thylakoids were only mildly solubilized and our procedure did not use Triton-extracted PSII enriched particles (BBY or G&Y preparations) as starting material, but whole thylakoids with stacked grana. We believe that this was important to preserve interaction of PsbS with its possible partners, including those not belonging to the PSII complex. Within Photosystem II–LHCII supercomplex, PsbS seems to interact with CP29 and various LHCII proteins (see Table 1). The association was confirmed by immuno-precipitation, as both CP29 and LHCII were pulled down from solubilized thylakoids by anti-PsbS antibody. Relatively strong solubilization of the membrane with deoxycholate weakened interaction (Fig. 4). Since the anti-PsbS antibody we used was highly specific, it is extremely unlikely that co-immunoprecipitation may be the result of unspecific cross-reaction with CP29 or LHCII. The data shown here confirm interaction of PsbS and LHCII, which has previously been hypothesized [12,33] and observed [16], although still not widely accepted. Pull down of CP29 by anti-PsbS antibody is in accordance with previous observations that PsbS is enriched in G&Y preparations containing PSII core and CP29. Indeed, it has been postulated on the basis of this information, that PsbS is located in the region where PSII core and CP29 interact [15]. Interaction between PsbS and CP29 may be a priori relevant for PsbS action during qE, but antisense inhibition of CP29 did not alter the ability of mutant plants to undergo this type of quenching [34]. On the contrary, association of PsbS with LHCII may play an important role during NPQ [7,12,16]. However, inhibition of a single Lhca or Lhcb antenna protein expression does not affect qE in field conditions but alters overall plant fitness [35]. Given that various LHCII components [34] have been found among the interaction partners of PsbS (Fig. 1C and Table 1), it is possible that these proteins can substitute each other when one of them is not expressed.

While the interaction of PsbS with components of the PSII–LHCII supercomplex was expected, to our knowledge, its association with other complexes has never been hypothesized before. Interaction of single membrane proteins with membrane-embedded complexes is often investigated by using co-immunoprecipitation in the field of protein import studies. For example, Tim50 which has a large domain in the intermembrane space has been shown to associate with Tim23 complex and to co-immunoprecipitate using antibodies against various members of the Tim23 complex [36]. In our case, a few points can be taken into account to rationalize the finding that PsbS interacts also with complexes other than PSII. The eluate of immunopurification is significantly enriched in PsbS: if PsbS was immuno-purified as part of a stable complex connected to the PSII core, we should also have found components of the PSII core in the eluate, which we did not. On the other hand, if PsbS was part of a relatively stable complex with CP29 or some LHCII components, we would expect a constant ratio of its amount in the purification eluate with respect to these proteins. Our data instead suggest that PsbS can be "extracted" relatively easily by the mild solubilization protocol used here, also as a single protein. Accordingly, PsbS was found to co-migrate on sucrose gradient with several fractions, containing LHCII, PSII core, and a mixture of PSII and PSI [16], when thylakoids were solubilized with 1% β-DM for 5 min on ice [37]. Furthermore, PsbS co-migrated with different complexes also in non-denaturing Deriphat-PAGE (Fig. 6). Similar results were observed in BN-PAGE as well (E.M. Aro, personal communication). These data, together with the result of Fig. 7, suggest that PsbS is relatively mobile in the plane of the membrane. Interestingly, the PSII minor antenna CP29 has recently been shown to be mobile as well and to be strongly associated with PSI when it becomes hyper-phosphorylated in the green alga *Chlamydomonas reinhardtii* [38]. In this organism, CP29 has been hypothesized to serve as a docking site for LHCII on PSI under state 2 conditions.

In the case of PsbS, there is no evidence reported in the literature in favour of any contribution of PsbS to state transition so the question remains open why PsbS associates with PSI. A complete lack of PSI-F prevents survival of transgenic plants [39]. *Arabidopsis thaliana* with 5% PSI-F suffered from chronic PSII photoinhibition. As a pure speculation, it may be envisioned that interaction of PSI-F with PsbS could avoid an increase of excitation pressure on PSII. It is worth observing that PSI-F down-regulated plants display a significantly changed thylakoid organization with distorted grana and no stroma lamellae [39]. If in higher plants changes in thylakoid organization may result in response to changes in irradiance (see e.g. [40]), it is tempting to speculate that interaction of PsbS with PSI-F, i.e. the tendency of PsbS to shuttle between PSII and PSI, may be the trigger to reorganization of thylakoids leading to photoprotection. This view is in agreement with a role of PsbS in influencing membrane dynamics, as suggested by Kiss et al. [41]. Concerning a possible, direct interaction of PsbS with PSI-L, it has to be mentioned that PSI-L together with PSI-K and PSI-A constitutes a binding pocket for a protein of the Lhc superfamily. Indeed the idea has been put forward that this pocket may be occupied by Lhcb proteins [42]. PsbS is also a member of the Lhc superfamily, although postulated to have four transmembrane domains [43].

ATP synthase complex, similarly to PSI, has recently been found in grana margins [44]. In this membrane region, as well as in stroma lamellae, interaction of PsbS with ATP synthase complex may occur. In our solubilization conditions, subunits of the F_1 headpiece of ATP synthase are still kept together (see Fig. 6). ATP synthase has been proposed as playing an essential role in qE [45,46]. Whether any interaction between PsbS and ATP synthase subunits might have a role during qE or in general during NPQ still remains an open question that deserves attention. While in the absence of PsbS, in *Arabidopsis* mutants, the *in vivo* midpoint potential of $Q_{(A)}$ [47] is altered, we could not find any indication in the literature about ATP synthase activity in these mutants.

The cytochrome b_6f complex has also been identified as a possible interaction partner of PsbS: the 3D structure of this complex from a thermophilic cyanobacterium (*Mastigocladus laminosus*) has recently been solved [48]. Two central cavities form at the interface of the dimer, where space might be sufficient to accommodate contact of PsbS with cytochrome b_6. The cytochrome b_6f complex is not expected to be dissociated under the solubilization conditions used here. In accordance, cytochrome f was also found in the eluate as revealed by Western blot (Fig. 3B) and could be immunoprecipitated by anti-PsbS antibody.

In summary, in this paper we provide multiple evidence that PsbS may interact, at least under some conditions, with various proteins of the PSII–LHCII supercomplex as well as with other photosynthetic complexes. Determination of the specific subunits responsible for the interaction with the different complexes is under way in our laboratory. Anyhow, the emerging picture is that PsbS has multiple locations within the plane of the membrane and may be preferentially associated to one or more complexes depending on the conditions.

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