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Proteomic approach to characterize the supramolecular organization of photosystems in higher plants

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Abstract

A project to investigate the supramolecular structure of photosystems was initiated, which is based on protein solubilizations by digitonin, protein separations by Blue native (BN)–polyacrylamide gel electrophoresis (PAGE) and protein identifications by mass spectrometry (MS). Under the conditions applied, nine photosystem supercomplexes could be described for chloroplasts of *Arabidopsis*, which have apparent molecular masses between 600 and 3200 kDa on BN gels. Identities of the supercomplexes were determined on the basis of their subunit compositions as documented by 2D BN/SDS–PAGE and BN/BN–PAGE. Two supercomplexes of 1060 and ~1600 kDa represent dimeric and trimeric forms of photosystem I (PSI), which include tightly bound LHCI proteins. Compared to monomeric PSI, these protein complexes are of low abundance. In contrast, photosystem II mainly forms part of dominant supercomplexes of 850, 1000, 1050 and 1300 kDa. According to our interpretation, these supercomplexes contain dimeric PSII, 1–4 LHCII trimers and additionally monomeric LHCII proteins. The 1300-kDa PSII supercomplex (containing four LHCII trimers) is partially converted into the 1000-kDa PSII supercomplex (containing two LHCII trimers) in the presence of dodecylmaltoside on 2D BN/BN gels. Analyses of peptides of the trypsinated 1300-kDa PSII supercomplex by mass spectrometry allowed to identify known subunits of the PSII core complex and additionally LHCII proteins encoded by eight different genes in *Arabidopsis*. Further application of this experimental approach will allow new insights into the supermolecular organization of photosystems in plants.
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1. Introduction

The photosynthetic electron transport system in chloroplasts is based on the presence of three protein complexes termed photosystem I (PSI), photosystem II (PSII) and cytochrome b_6f complex (b_6f complex). Furthermore, light harvesting complexes (LHCI and LHCII) are associated with the two photosystems to increase the rates of their primary photoreactions. Linear photosynthetic electron transport involves PSII, the b_6f complex and PSI, and catalyses reduction of ferredoxin, thioredoxin or $NADP^+$ by water oxidation in the light. At the same time, linear electron transport causes formation of a proton gradient across the thylakoid membrane which is the prerequisite for "photophosphorylation", the light-driven phosphorylation of ADP by the ATP synthase complex. Besides linear electron transport, PSI, the b_6f complex and possibly in addition a NADH dehydrogenase complex can carry out cyclic electron transport which only contributes to the proton gradient across the thylakoid membrane but does not lead to reduction or oxidation of external compounds.

Extensive knowledge is available on the structures of the two photosystems and the b_6f complex. Using X-ray

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crystallography, structures of PSI from *Synechococcus elongatus* and pea (Jordan et al., 2001; Ben-Shem et al., 2003), the structures of the b_6f complex from the cyanobacterium *Mastigocladus laminosus* and *Chlamydomonas* (Stroebel et al., 2003; Kurisu et al., 2003) and the structures of photosystem II from the cyanobacteria *Synechococcus elongates* and *Prochloron didemni* (Zouni et al., 2001; Bibby et al., 2003) were resolved. Furthermore, the structure of LHCII was solved by electron crystallography (Kühlbrandt et al., 1994; Liu et al., 2004).

In contrast, the supermolecular organization of protein complexes involved in photosynthesis is less understood. However, fascinating insights into supercomplexes formed by photosystems were obtained by electron microscopy combined with computer image analyses: in cyanobacteria, the photosystem I forms trimers (Kruip et al., 1993), which are surrounded by an antenna ring formed of 18 copies of the antenna protein CP43' under iron-deficiency conditions (Boekema et al., 2001a; Bibby et al., 2001). In contrast, PSI from *Chlamydomonas* and higher plants is monomeric and asymmetrically binds LHCI proteins on one side of the complex (Boekema et al., 2001b; Germano et al., 2002; Kargul et al., 2003). Four to 14 LHCI proteins are estimated to be associated with individual PSI complexes in plants. In contrast, photosystem II is dimeric in cyanobacteria and higher plants (Boekema et al., 1995). It is associated with monomeric and trimeric LHCII proteins. There are 2× three binding sites for LHCII trimers at dimeric PSII, which are termed S-, M- and L-positions according to their binding strength for LHCII trimers (S, strong; M, moderate and L, loose) (Boekema et al., 1998a,b, 1999a,b; Yakushevska et al., 2001). According to a nomenclature proposed by Boekema et al. (1998a), which was extended by Yakushevska et al. (2001), the PSII supercomplexes are termed by the binding positions of dimeric PSII core complex (C_2) filled with LHCII trimers (C_2S_2: dimeric PSII associated with two LHCII trimers at the S position, $C_2S_2M_2$: dimeric PSII associated with two LHCII trimers at the S position and two LHCII trimers at the M position, and so on). Additionally, monomeric LHCII proteins bind to the photosystem II supercomplexes (Boekema et al., 1999b). However, the structures of PSII supercomplexes in plants are still a matter of debate and possibly will not be precisely resolved until availability of X-ray crystallography data.

Recently, a novel electrophoretic strategy was developed to characterize supercomplexes of the respiratory chain in mitochondria, which is based on protein solubilization using mild non-ionic detergents and separation of the solubilized protein complexes by Blue-native (BN) gel electrophoresis (Schägger and Pfeiffer, 2000). Using this approach, distinct supercomplexes, formed of respiratory complexes I + III, III + IV and I + III + IV, could be described for mitochondria from yeast, mammals and plants (Schägger and Pfeiffer, 2000; Schägger, 2001a, 2002; Zhang et al., 2002; Eubel et al., 2003, 2004; Pfeiffer et al., 2003).

BN–PAGE was previously employed to characterize photosystems in higher plants (Kügler et al., 1997). Using protein solubilizations with dodecylmaltoside, monomeric photosystems I and II, dimeric b_6f complex and trimeric LHCII complex from spinach, tobacco and potato chloroplasts were resolved by BN–PAGE (Kügler et al., 1997, 1998; Singh et al., 2000). Additionally, photosystem II supercomplexes could be described by this experimental approach in pea and tobacco (Thidholm et al., 2002; Surosa et al., 2004). Recently, protein solubilization by digitonin was combined with BN–PAGE and allowed to visualize supercomplexes of photosystems in *Chlamydomonas* (Rexroth et al., 2003).

Here, we describe a proteomic approach to characterize photosystem supercomplexes of *Arabidopsis* using digitonin solubilizations, BN–PAGE and mass spectrometry. Nine photosystem supercomplexes are visible in the molecular mass range between 600 and 3200 kDa. The identities of these supercomplexes were analysed by 2D BN/SDS–PAGE and 2D BN/BN–PAGE combined with mass spectrometry. Two supercomplexes of 1060 and ~1600 kDa represent dimeric and trimeric PSI. Four supercomplexes of 850, 1000, 1150 and 1300 kDa represent dimeric PSII, which are associated with a varying number of LHCII trimers and probably are of C_2S_1 (or C_2M_1), C_2S_2 (or $C_2S_1M_1$ or C_2M_2), $C_2S_2M_1$ (or $C_2S_1M_2$) and $C_2S_2M_2$ structures. Trypsination of the $C_2S_2M_2$ supercomplex, separation of the generated peptides by liquid chromatography (LC) and analysis of the separated peptides by electrospray tandem mass spectrometry (ESI–MS/MS) allowed to identify more than 20 different subunits of PSII, including eight different LHCII proteins. Applications of this experimental approach are discussed.

2. Results

2.1. Identification of chloroplast supercomplexes of Arabidopsis by 1D Blue-native PAGE

Digitonin proved to be a very suitable detergent for the solubilization and stabilization of supercomplexes of *Arabidopsis* mitochondria (Eubel et al., 2003, 2004). We therefore started a project to systematically investigate protein complexes of chloroplasts using this detergent. To determine the optimal detergent–protein ratio for supercomplex solubilization, isolated *Arabidopsis* chloroplasts were treated with different digitonin concentrations and subsequently analysed by 1D Blue-native PAGE. Detergent–protein ratios of 1 g/g or below only

allowed insufficient solubilization of chloroplast protein complexes, whereas ratios of 1.5 g/g or higher gave very good results (data not shown). Therefore, all further experiments were carried out using 1.5 g digitonin per g protein. Under these conditions, 14 protein complexes can be resolved on 1D Blue-native gels (Fig. 1). Due to the presence of Coomassie dyes during gel electrophoresis, protein bands become visible without staining (Fig. 1A). However, subsequent staining of 1D gels by Coomassie blue significantly increased the visibility of protein complexes (Fig. 1B).

To determine the apparent molecular masses of the resolved chloroplast protein complexes, mitochondrial protein complexes from *Arabidopsis* were separated in parallel by 1D BN–PAGE (Fig. 1). Five complexes could be detected, which were identified by comparison to gels published previously (Eubel et al., 2003): the F_1 part of the mitochondrial ATP synthase complex (390 kDa), dimeric complex III (500 kDa), monomeric complex V (600 kDa), monomeric complex I (1000 kDa) and a supercomplex formed of dimeric complex III and complex I (1500 kDa). Using these mitochondrial protein complexes as a molecular mass standard, the sizes of chloroplast complexes could be determined to lie in the molecular mass range between 110 and 3200 kDa (Table 1). [However, sizes above 1500 kDa and below 390 kDa should be considered with caution, because they are not covered by the mitochondrial molecular mass standard. Furthermore, separations by Blue-native PAGE not necessarily reflect exactly calculated molecular masses as reported before (Schägger, 2001b).]

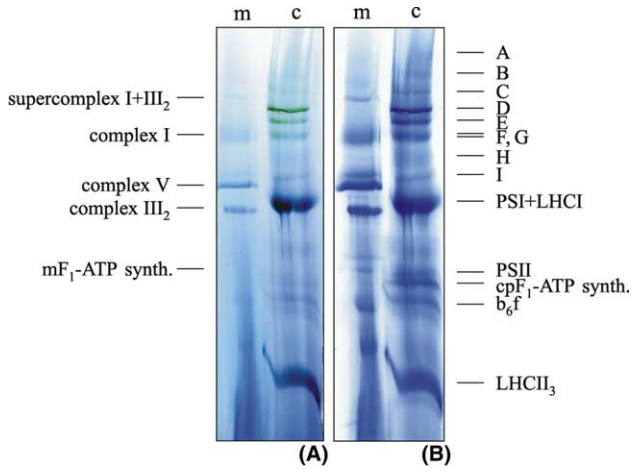

Fig. 1. Separation of mitochondrial (m) and chloroplast (c) protein complexes and supercomplexes of *Arabidopsis* by 1D BN–PAGE. Proteins were solubilized by 1.5 g/g digitonin. (A) Unstained gel; (B) Coomassie-stained gel. Identities of mitochondrial protein complexes are given to the left and identities of known chloroplast protein complexes to the right (mF_1-ATP synth., F_1 part of the mitochondrial ATP synthase complex; complex III_2, dimeric cytochrome *c* reductase; complex V, mitochondrial ATP synthase; complex I, NADH dehydrogenase; supercomplex I + III_2, supercomplex formed of dimeric cytochrome c redutase and NADH dehydrogenase; $LHCII_3$, trimeric light harvesting complex II; b_6f, cytochrome b_6f complex; cpF_1-ATP synth., F_1-part of plastidic ATP synthase; PSII, photosystem II; PSI, photosystem I; LHCI, light harvesting complex I). Unknown chloroplast protein complexes are designated by capital letters (A–I). Apparent molecular masses of all protein complexes are given in Table 1.

Table 1
Apparent molecular masses of digitonin solubilized mitochondrial and chloroplast protein complexes on BN gels[a]

Complex	Molecular mass (kDa)
Mitochondria	
Supercomplex I + III_2	1500
Complex I	1000
Complex V	600
Complex III_2	500
mF_1-ATP synthase	390
Chloroplasts	
A	~3200
B	~2200
C	~1600
D	300
E	1150
F	1060
G	1000
H	850
I	650
PSI + LHCI	530
PSII	~370
cpF_1-ATP synthase	~350
Dimeric b_6f complex	~300
$LHCII_3$	~110

[a] The designations of the complexes correspond to Fig. 1.

Identities of the chloroplast protein complexes smaller than 530 kDa can be predicted by comparison to previous separations of dodecylmaltoside solubilized chloroplast protein complexes from spinach and tobacco on the basis of 1D BN–PAGE (Kügler et al., 1997). In contrast, nine protein complexes between 650 and ~3200 kDa are of unknown identity (termed complexes A–I in Fig. 1).

2.2. Characterization of chloroplast supercomplexes by 2D BN/SDS–PAGE

One-dimensional Blue-native gels were combined with SDS–PAGE to elucidate identities of the resolved protein complexes on the basis of subunit compositions. For comparison, protein solubilizations were carried out with digitonin or dodecylmaltoside, because 2D BN/SDS gels of dodecylmaltoside-treated chloroplast protein complexes from spinach and tobacco were characterized previously in detail (Kügler et al., 1997). Indeed, 2D BN/SDS gels of dodecylmaltoside solubilized protein complexes of *Arabidopsis* chloroplasts very much resemble gels shown before for spinach and tobacco (Fig. 2A, Kügler et al., 1997). The largest protein

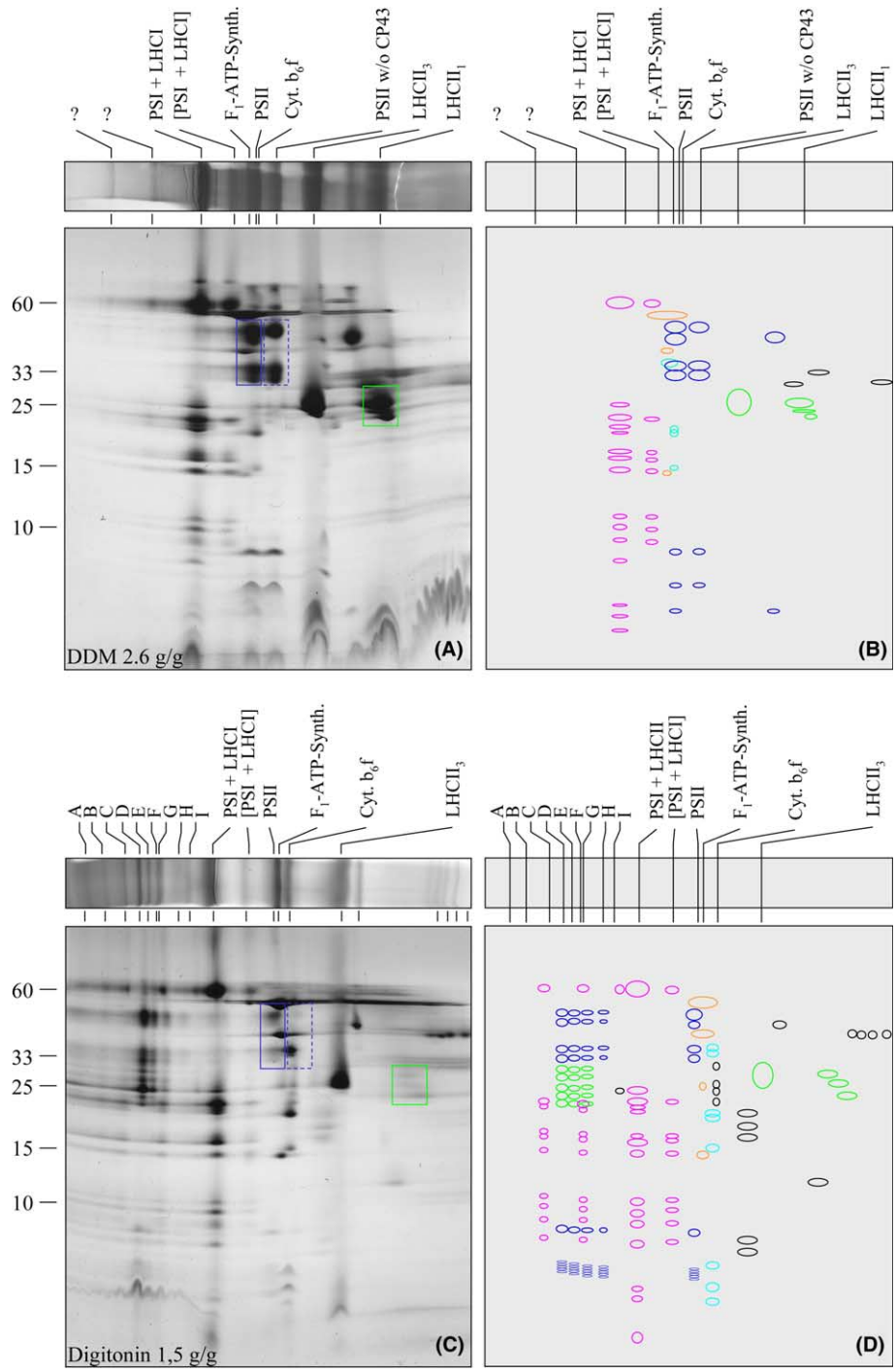

Fig. 2. Two-dimensional resolution of chloroplast protein complexes and supercomplexes of *Arabidopsis* by BN/SDS–PAGE. (A) Proteins were solubilized by 2.6 g/g dodecylmaltoside (B: scheme of the gel in A); (C) Proteins were solubilized by 1.5 g/g digitonin (D: scheme of the gel in C). Gels were Coomassie-stained. Identities of protein complexes are given above the gels (for designations see legend of Fig. 1; [PSI + LHCI], subcomplex of PSI + LHCI lacking some LHCI proteins). Subunits of individual protein complexes are given in colours on the scheme to facilitate recognition of protein complexes (purple: subunits of photosystem I or LHCI; blue: subunits of photosystem II; light blue: subunits of the b_6f complex; orange: subunits of F_1-ATP synthase; green: subunits of LHCII; black: subunits of unknown protein complexes. The numbers to the right refer to molecular masses of standard proteins (in kDa). Proteins boxed in green on the gels in A and C indicate monomeric LHCII and proteins boxed in blue the CP47/CP43/D2 and D1 proteins of monomeric photosystem II, which are well visible upon dodecylmaltoside solubilization but hardly detectable upon digitonin solubilization.

complex represents photosystem I together with LHCI proteins (530 kDa). A smaller version of this complex, which lacks some LHCI proteins in the 20–25 kDa range, has slightly higher electrophoretic mobility. The four protein complexes in the 250–400 kDa range represent the F_1 part of plastidic ATP synthase, monomeric

PSII, a subcomplex of monomeric PSII lacking the CP43 protein and dimeric b_6f complex. Furthermore, trimeric and monomeric LHCII are resolved at ~110 and ~30 kDa.

In contrast, 2D BN/SDS gels of digitonin solubilized chloroplast proteins from *Arabidopsis* considerably differ in comparison to gels of dodecylmaltoside solubilized proteins of the same fraction (Fig. 2C). Most strikingly, a significant amount of protein forms part of supercomplexes larger than 530 kDa. At the same time, proteins and protein complexes below 530 kDa are significantly reduced: (i) monomeric LHCI is hardly detectable, (ii) trimeric LHCII is reduced, (iii) the subcomplex of PSII lacking CP43 is absent, (iv) monomeric PSII is very much reduced and (v) the subcomplex of monomeric PSI lacking LHCI proteins is less abundand.

The subunit compositions on 2D BN/SDS gels allow to identify the newly described supercomplexes (Fig. 2C): The 1300-, 1150-, 1000- and 850-kDa complexes D, E, G and H represent PSII supercomplexes containing decreasing amounts of LHCII proteins and the ~1600, 1060 and 650 kDa complexes C, F and I represent PSI supercomplexes associated with LHCI. PSII supercomplexes are very abundant, whereas the PSI supercomplexes are of lower abundance. Identity of the 3200 and 2200 kDa supercomplexes A and B is unclear, because their subunits are hardly detectable on the 2D gel in Fig. 2C, but more likely represent PSI supercomplexes than PSII supercomplexes.

2.3. Characterization of chloroplast supercomplexes by 2D BN/BN–PAGE

To better understand the composition of the supercomplexes A–I, a novel 2D gel electrophoresis procedure was applied, which employs BN–PAGE in the presence of digitonin in the first gel dimension and BN–PAGE in the presence of low dodecylmaltoside concentrations in the second dimension (Schägger and Pfeiffer, 2000). All protein complexes and supercomplexes likewise stable in the presence of both detergents are positioned on a diagonal line on the resulting 2D gels, whereas supercomplexes destabilized in the presence of dodecylmaltoside are dissected into protein complexes of higher electrophoretic mobility.

BN/BN–PAGE of digitonin solubilized chloroplast protein complexes from *Arabidopsis* (Fig. 3) supports the results obtained by BN/SDS–PAGE. Most PSI is present in a monomeric form which includes LHCI proteins (530 kDa). The PSI supercomplexes C and F (~1600 and 1060 kDa) are of low abundance and partially dissected into monomeric PSI on the BN/BN gels. Therefore, these complexes most likely represent trimeric and dimeric forms of PSI. The 650-kDa supercomplex of PSI seems to represent monomeric PSI associated with LHCII. In contrast to the three PSI supercomplexes, the 1000-kDa PSII supercomplex proved to be stable during BN/BN–PAGE. Furthermore, the 1300- and 1150-kDa supercomplexes of PSII are partially dissected into the 1000-kDa supercomplex and LHCII trimers on the

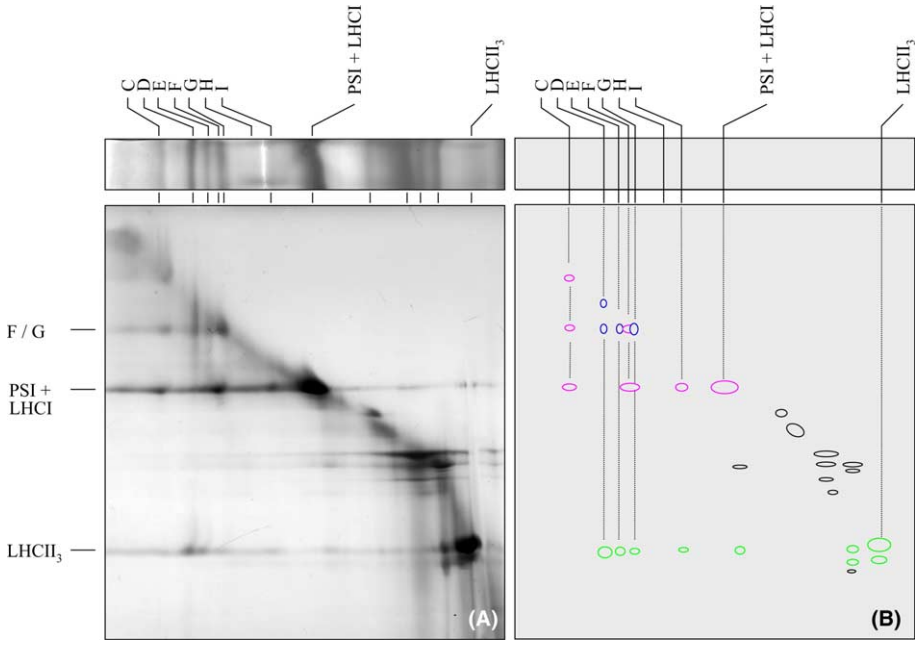

Fig. 3. Two-dimensional resolution of chloroplast protein complexes and supercomplexes of *Arabidopsis* by BN/BN–PAGE. Proteins were solubilized by 1.5 g/g digitonin. (A) Coomassie-stained gel; (B) scheme of the gel in (A). Identities of protein complexes are given above and to the left of the gel (for designations see legend of Fig. 1) and by a colour code (see legend of Fig. 2).

Table 2
Identified subunits of the 1300-kDa PSII supercomplex D[a]

No.	Gene	Identity	Score
1.	psbB	CP47 subunit of PSII	509
2.	At4g10340	CP26 subunit of PSII (Lhcb5)	432
3.	At1g15820	CP24 subunit of PSII (Lhcb6)	348
4.	psbC	CP43 subunit of PSII	262
5.	At5g66570	33 kDa subunit of OEC	253
6.	At3g08940	CP29 subunit of PSII (Lhcb4.1)	244
7.	psbA	D1 subunit of PSII	244
8.	At5g01530	CP29 subunit of PSII (Lhcb4.2)	240
9.	At2g05070	LHCII antenna protein (Lhcb2.2)	204
10.	At3g50820	33 kDa subunit of OEC	201
11.	At1g29910	LHCII antenna protein (Lhc1)	199
12.	At2g34430	LHCII antenna protein (Lhcb1)	189
13.	At1g31330	putative subunit III of PSI	165
14.	At3g47470	CP29 subunit of PSII (Lhcb4.3)	150
15.	psbD	D2 subunit of PSII	144

[a] Identifications are based on LC–MS/MS.

second gel dimension (Fig. 3). The 850-kDa PSII supercomplex, which is of lower abundance (Fig. 1), was not detectable on the BN/BN gels.

2.4. Identification of the subunits of the 1300-kDa PSII supercomplex D by mass spectrometry

The 1300-kDa PSII supercomplex is of very high abundance, comprises more than 50% of total PSII and includes the largest amounts of LHCII proteins. In order to precisely define the subunits present in this supercomplex, a protein band representing this supercomplex was cut out of a 1D BN gel and analysed by mass spectrometry. Peptides generated by trypsination of the supercomplex were separated by LC and subsequently peptides were identified by tandem mass spectrometry as described in Section 4. Thirty different proteins could unambiguously be matched to specific gene products encoded by the *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2000). Depending on the number of peptides identified per gene product, identified proteins have MASCOT scores between 35 and 509. At least 14 of the 15 identified proteins with the highest MASCOT scores (>150) encode subunits of PSII or LHCII (Table 2): the CP47, CP43, D1 and D2 proteins, two isoforms of the 33 kDa subunit of the oxygen evolving complex and eight different LHCII proteins. Further subunits of the PSII supercomplex were also unambiguously identified but have lower MASCOT scores (<100), e.g., the PsbL, PsbH proteins and the α- and β-subunit of cytochrome b_{559} (data not shown). The identified LHCII subunits exhibit sequence similarity to LHCII proteins from other organisms and can be assigned to five of the six subclasses which were defined for the LHCII protein family (termed Lhcb1–Lhcb6). However, assignments are partially difficult, because more than 30 genes of *Arabidopsis* encode LHC proteins, which have very similar amino acid sequences (The Arabidopsis Genome Initiative, 2000).

3. Discussion

3.1. Protein supercomplexes in chloroplasts

Digitonin not only is a very suitable detergent for the solubilization and stabilization of supercomplexes from mitochondria, but also proved to be a powerful tool for supercomplex characterization in chloroplasts. In combination with BN–PAGE, nine photosystem supercomplexes can be resolved. Four of these represent PSII supercomplexes, three PSI supercomplexes and two are of so far unknown identity. Mitochondria were shown to contain heteromeric supercomplexes, which comprise more than one type of protein complex (Schägger and Pfeiffer, 2000; Eubel et al., 2003). In contrast, all described chloroplast protein supercomplexes contain only one type of photosystem, which is associated with light harvesting complexes. However, this result is not unexpected because the two photosystems are known to be localized in different areas of thylakoid membranes, PSI mainly in unstacked and PSII mainly in stacked membrane regions. The cytochrome b_6f complex, which always is dimeric for functional reasons, and the ATP synthase complex were not part of supercomplexes under all conditions applied. In contrast, mitochondrial ATP synthase forms dimers and the mitochondrial bc_1 complex forms supercomplexes with cytochrome *c* oxidase and/or NADH dehydrogenase.

3.2. PSI supercomplexes

About 90% of PSI is in monomeric state after solubilization using dodecylmaltoside or digitonin and sep-

aration by BN–PAGE (Figs. 1–3). In the presence of either detergent, it forms a band at 530 kDa and includes LHCI proteins. These data are very much in line with the results published for the crystallized PSI from pea, which has a molecular mass of 525 kDa and includes two tightly bound LHCI dimers (Ben-Shem et al., 2003). A minor form of PSI on the 1D BN gels is slightly smaller and seems to lack some LHCI proteins. This form of PSI is more abundant in the presence of dodecylmaltoside, indicating that it probably is formed by degradation of PSI + LHCI during solubilization. Furthermore, upon digitonin solubilization, about 10% of PSI forms part of three supercomplexes of 650, 1060, ~1600 kDa (Fig. 2C; very low amounts of the 1060- and 1600-kDa PSI supercomplexes are also visible upon dodecylmaltoside solubilization on BN gels; Fig. 2A). The two larger PSI supercomplexes most likely represent trimeric and dimeric forms of PSI + LHCI, because their molecular masses exceed the one of monomeric PSI by factor 2 or 3 and because they both are partially dissected into monomeric PSI complexes on 2D BN/BN gels (Fig. 3). Dimeric and trimeric PSI were previously described for higher plants, but found to be of low abundance (Boekema et al., 2001b). However, it is speculated that these supercomplexes possibly are artificially formed during solubilization, because image analyses of EM data revealed irregular interactions of the monomers and because of theoretical considerations on the basis of the crystal structure of pea PSI (Boekema et al., 2001b; Ben-Shem et al., 2003). On the other side, there are no hints for other artificial supercomplex formations on BN gels between any of the known chloroplast protein complexes under the conditions applied. Therefore, the occurrence of dimeric and trimeric PSI complexes in higher plants can currently not be excluded.

The 650 kDa complex includes PSI, LHCI proteins and additionally another protein component, which dissociates on the second dimension of BN/BN gels and co-migrates with trimeric LHCII (Fig. 3). It previously was reported that phosphorylated forms of LHCII are detached from PSII and transferred to PSI to regulate light distribution between the two photosystems in chloroplasts (Lunde et al., 2000).

3.3. PSII supercomplexes

Monomeric PSII is very abundant on BN gels upon dodecylmaltoside solubilization, but hardly detectable after treatment of chloroplast fractions with digitonin. Furthermore, monomeric LHCII is nearly absent after digitonin treatment, trimeric LHCII is reduced and a subcomplex of dimeric PSII lacking the CP43 protein, which clearly is visible after dodecylmaltoside solubilizations on BN gels, is absent. Instead, about 90% of PSII in digitonin treated fractions forms part of the four supercomplexes of 850, 1000, 1150 and 1300 kDa, the latter of which is most abundant.

The apparent molecular mass of monomeric PSII on BN gels differs slightly upon dodecylmaltoside and digitonin solubilizations: in dodecylmaltoside fractions it migrates faster than the F_1 ATP synthase, whereas in digitonin fractions it migrates slower. Indeed, for unknown reasons, the apparent molecular mass of monomeric PSII is rather high in the presence of digitonin and lies in the range of 370 kDa. We speculate that the 850, 1000, 1150 and 1300 kDa supercomplexes represent dimeric PSII supercomplexes associated with one, two, three or four LHCII trimers and additional monomeric LHCII proteins. A dimeric structure of PSII was previously shown by EM (Boekema et al., 1995), X-ray crystallography (Zouni et al., 2001; Bibby et al., 2003) and genetic investigations (Swiatek et al., 2001). Furthermore, dimeric PSII was shown to be associated with 1–4 trimeric LHCII complexes and additional monomeric LHCII proteins by image analyses of EM data (Boekema et al., 1999b; Yakushevska et al., 2001). There are 2× three binding sites for LHCII trimers in higher plants, which differ with respect to their binding strength for LHCII trimers and which were designated S, M and L positions as given in Section 1. For *Arabidopsis*, C_2S, $C_2S_2/C_2S_1M_1/C_2M_2$, $C_2S_2M_1/C_2S_1M_2$ and $C_2S_2M_2$ complexes could be described (Yakushevska et al., 2001). The presence of 850, 1000, 1150 and 1300 kDa PSII supercomplexes on BN gels nicely corresponds to the EM data. In fact, these four supercomplexes comprise increasing amounts of chlorophyll, as visible in Fig. 1A. The size difference of 150 kDa between the individual PSII supercomplexes can be explained by one LHCII trimer (about 110 kDa) and additionally 1–2 monomeric LHCII proteins. Indeed it was reported previously that binding of LHCII trimers to dimeric PSII is preceeded/accompanied by binding of distinct LHCII monomers (Boekema et al., 1999b).

Interestingly, the 1300-kDa supercomplex, which probably has $C_2S_2M_2$ composition, is the most abundant PSII supercomplex in digitonin solubilized fractions, whereas the 1000-kDa C_2S_2 (or $C_2S_1M_1/C_2M_2$) complex is the most stable PSII supercomplex which is not dissected during BN/BN–PAGE (Fig. 3).

PSII supercomplexes were not visible on BN gels upon dodecylmaltoside solubilizations under the conditions applied (Fig. 2B), most likely because this detergent destabilized these supramolecular structures. However, PSII supercomplexes could be described at lower dodecylmaltoside concentrations (Boekema et al., 1995). They also were detected previously on 1D BN gels representing minor forms of PSII complexes in pea (Thidholm et al., 2002). Furthermore, similar protein complexes are visible on 2D BN/SDS gels of digitonin solubilized chloroplast fractions from *Chlamydomonas* (Rexroth et al., 2003). Table 3 summarizes our data on

Table 3
Composition of digitonin solubilized chloroplast supercomplexes resolved on BN gels

Supercomplex[a]	Apparent molecular mass (kDa)	Identity and proposed composition[b]
A	~3200	?
B	~2200	?
C	~1600	$[PSI + LHCI]_3$
D	1300	$[PSII]_2$: $C_2S_2M_2$
E	1150	$[PSII]_2$: $C_2S_2M_1$ or C_2 S_1M_2
F	1060	$[PSI + LHCI]_2$
G	1000	$[PSII]_2$: C_2S_2 or $C_2S_1M_1$ or C_2M_2
H	850	$[PSII]_2$: C_2S_1 or C_2M_1
I	650	$[PSI + LHCI]_1 + LHCII$
	530	$[PSI + LHCI]_1$

[a] Designations of the supercomplexes corresponds to Figs. 1–3 and Table 1.
[b] Nomenclature of PSII supercomplexes according to Boekema et al. (1998a) and Yakushevska et al. (2001).

the nine chloroplast supercomplexes from *Arabidopsis* visible on BN gels and their proposed composition.

3.4. Outlook

BN–PAGE originally was developed to characterize the protein complexes of the respiratory chain (Schägger and von Jagow, 1991) but also is a very powerful tool for investigations on chloroplast protein complexes (Kügler et al., 1997). In combination with digitonin solubilizations, supercomplexes can be stabilized for both cellular compartments. The availability of efficient protein identification procedures, which are based on mass spectrometry, now offers new strategies to characterize supercomplexes. So far, the exact composition of PSII supercomplexes with respect of LHCII proteins is largely unknown, because their structures are too labile to allow X-ray crystallography. Analysis by mass spectrometry now allowed to identify eight different LHCII proteins to be present in the 1300-kDa PSII supercomplex of *Arabidopsis*. The experimental strategy described in our study will allow to give new insights into the subunit compositions of labile supermolecular structures in plant organelles.

4. Experimental

4.1. Isolation of chloroplasts and mitochondria from Arabidopsis

Arabidopsis thaliana var. Columbia was grown for about 3 weeks at 24–26 °C/10,000 lux at long day conditions. Starting material for chloroplast preparations were about 200 g of plants (leaves and stems). Tissue was homogenized in extraction buffer (330 mM mannitol, 30 mM Hepes, 2 mM EDTA, 3 mM $MgCl_2$ and 0.1% (w/v) BSA, pH 7.8) by a Waring blender for 3× 3 s and filtered through four layers of gaze. Subsequently, chloroplasts were sedimented by centrifugation for 3 min at 2000*g*/4 °C and purified by Percoll density gradient centrifugation (40% [v/v] Percoll in extraction buffer) for 30 min at 30,000*g*/4 °C. Intact chloroplasts are represented by the lowest green band of the gradients. They are removed by a Pasteur pipette, supplemented with 4 volumes of extraction buffer without BSA and sedimented by centrifugation for 10 min at 2500*g*/4 °C. Finally chloroplasts are resuspended in extraction buffer without BSA at a protein concentration of 15 mg/ml, frozen by liquid nitrogen and stored at −80 °C. Mitochondria from *A. thaliana* cell suspension cultures were isolated as outlined previously (Werhahn et al., 2001).

4.2. Solubilization of membrane proteins

Purified organelles (750 μg protein) were sedimented by centrifugation for 10 min at 1250*g* (chloroplasts) or 14,300*g* (mitochondria) at 4 °C and resuspended in one of the two following buffers:

- 80 μl "digitonin solubilization buffer" (30 mM Hepes, pH 7.4, 150 mM potassium acetate, 10% glycerol, 2 mM PMSF and 1.5% [w/v] digitonin [Fluka, Buchs, Switzerland]);
- 95 μl "dodecylmaltoside solubilization buffer" (750 mM aminocaproic acid, 50 mM Bis–Tris, pH 7.0, 0.5 mM EDTA, 1 mM PMSF and 2% (w/v) dodecylmaltoside [Roche, Mannheim, Germany]).

Samples solubilized with digitonin were incubated for 20 min on ice and afterwards centrifuged for 30 min at 15,000*g*/4 °C, whereas samples solubilized with dodecylmaltoside were directly centrifuged at the same conditions. Supernatants were supplemented with 20 μl Coomassie-blue solution (5% [w/v] Coomassie-blue in 750 mM aminocaproic acid) and directly loaded onto Blue-native gels.

4.3. Gel electrophoresis

One-dimensional BN–PAGE and 2D BN/SDS–PAGE were carried out as described by Schägger (2001b) and 2D BN/BN–PAGE as outlined by Schägger and Pfeiffer (2000).

4.4. Protein identifications by mass spectrometry (LC–MS/MS)

A band of a 1D Blue-native gel containing the 1300-kDa PSII supercomplex D was excised, sliced in 1-mm^3 cubes, and incubated once in 200 μl of 50 mM NH_4HCO_3 and twice in 200 μl of 50% acetonitril/25 mM NH_4HCO_3 for 30 min. Subsequently, the protein-containing gel pieces were dried in a SpeedVac concentrator, rehydrated in 2 μg/ml sequencing-grade modified porcine trypsin (Promega, Madison, WI) in 50 mM NH_4HCO_3 and incubated overnight at 37 °C. The resulting peptides were collected by two successive extractions with 50 mM NH_4HCO_3 and 50% acetonitrile/0.5% formic acid. Extracts were pooled in a microcentrifuge tube and lyophilized in a SpeedVac concentrator.

For LC–MS analysis the peptides were resuspended in 0.1% trifluoroacetic acid. About 10 μl of each sample was injected into the Ultimate Nano-HPLC (LC Packings/Dionex) and separated on a C_{18} reversed-phase column (75 μm, 150 mm, PepMap, LC Packings), using a 120-min gradient (0–60% B (A = 0.1% formic acid; B = 60% acetonitrile/0.1% formic acid) and a flowrate of 200 nl/min. Doubly and triply charged peptide-ions were automatically selected by the MassLynx software and fragmented in a Q-TOF2 mass spectrometer (Micromass, Manchester, UK).

MS/MS-fragmentation data were analysed using an internal MASCOT-server (Matrix Science) (Perkins et al., 1999) searching against the NCBI-*A. thaliana* database. For the identification of proteins, the MASCOT default significance criteria were used, which means that if the score for a particular match exceeds the significance level, there is less than a 1 in 20 chance that the observed match is a random event. In the case of the *A. thaliana* database this significance threshold score was set to >34 by Mascot. The protein scores in the result table (Table 2) were generated cumulatively by the corresponding peptide scores.

It should be noted that not all proteins can be identified by this experimental approach, because some proteins lack basic amino acids and therefore are not fragmented by trypsin or because trypsin fragmentation in some cases only gives rise to hydrophobic peptides, which easily are lost during peptide purification prior to mass spectrometry.

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