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Assembly of Photosystem II in chloroplast *psbEFLJ* operon mutants

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Introduction

Cytochrome b_{559} (Cyt b_{559}) is a membrane-embedded heme protein of photosystem II, consisting of a α subunit (9 kDa) and a β subunit (4 kDa), which are encoded by chloroplast *psbE* and *psbF* genes, respectively. In higher plants and in cyanobacteria, these genes form the *psbEFLJ* operon. Deletion of the *psbE* gene from *Chlamydomonas* and the *psbF* gene from *Synechocystis* resulted in a loss of PSII, detected by oxygen evolution assays and variable fluorescence measurements (Morais et al., 1998, Pakrasi et al., 1990, respectively). Deletion of the *psbL* gene in *Synechocyctis* also led to a loss of PSII function (Anbudurai and Pakrasi, 1992). Replacement of the *psbJ* gene by a stop codon in *Synechocystis*, on the other hand, resulted in mutants capable of photoautotrophic growth. However, the growth of the mutants was considerably slower and the PSII-mediated oxygen evolution was less than half of that of the wild type cells (Lind et al., 1993).

Here we have utilized the blue-native gel electrophoresis (BN-PAGE) to address the role of the α and β subunits of the Cyt b_{559}, and particularly that of the low-molecular weight proteins PsbL and PsbJ in the assembly process of photosystem II complexes in higher plants.

Materials and methods

Tobacco *(Nicotiana tabacum* L.*)* chloroplasts were transformed using biolistic particle bombardment of leaves. Four mutants were constructed, each having one *psbEFLJ* operon gene inactivated. In addition, two controls, wild type (WT) and a line where only a selective marker has been inserted (RV), were used.

Thin sections of leaves for electron microscopy were prepared using standard methods and samples were examined with a Jeol Jem 1200EX electron microscope. Thylakoid membranes were isolated as described in Baena-Gonzales et al. (1999) and chlorophyll was quantitated according to Porra et al. (1989). To analyze the composition of thylakoid membrane protein complexes, blue-native electrophoresis was carried out according to Kugler et al. (1997) and Schägger et al. (1994) with slight modifications. The polypeptide composition was further analyzed by SDS-PAGE (Laemmli, 1970) as second dimension (see figure legends for details). The protein components in various complexes were identified with mass-spectrometry analysis (MALDI). For more sensitive analysis of the composition of various PSII assemblies, the second-dimension gels were analysed by Western blotting.

Results

Chloroplast ultrastructure

The thylakoid membrane ultrastructure was severely altered in $\Delta psbE$ and $\Delta psbF$ mutants (Fig. 1). The shape of the chloroplasts was strikingly affected, as the chloroplasts were swollen to round-shaped form. Moreover, the thylakoid membrane network was strongly reduced and the amount of thylakoids per granum was significantly lower than in controls. In $\Delta psbL$ and $\Delta psbJ$ mutants the shape of chloroplasts resembled that of the wild-type. However, the grana stacks were composed of fewer thylakoids as compared to the wild-type chloroplasts.
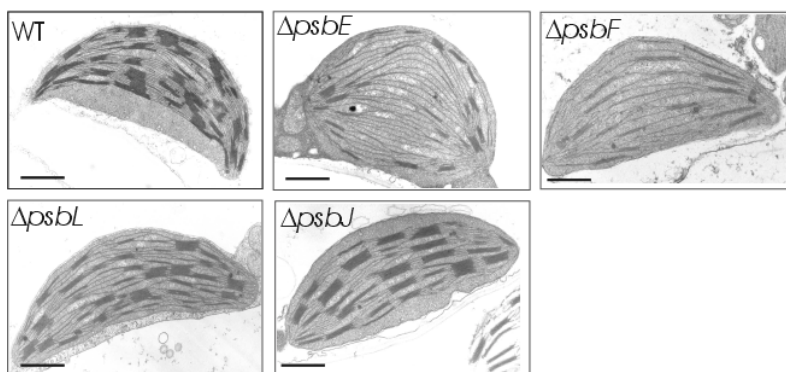

Fig. 1. Electron micrographs (10 000 x) of *psbEFLJ* operon mutant chloroplasts. The bar indicates 1 µm.
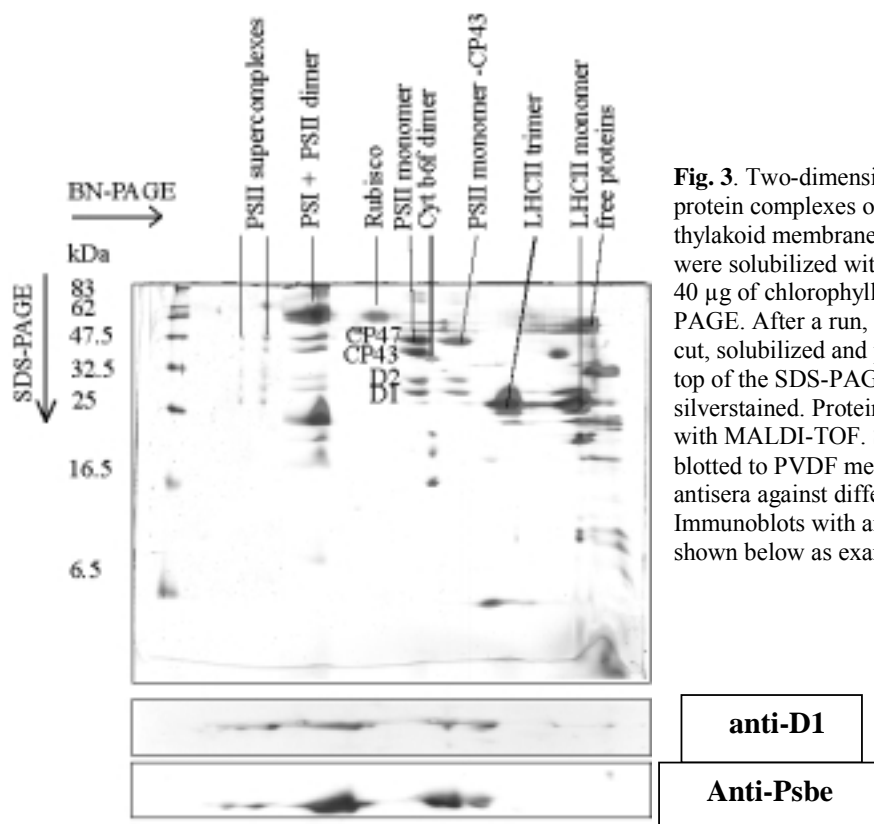
Assembly of thylakoid *membrane protein complexes*

psbE, $\Delta psbF$ and $\Delta psbL$ mutants showed the absence of intact PSII monomers (PSII core composed of D1, D2, Cyt b_{559}, CP47 and CP43) when analysed with one-dimensional BN-PAGE (Fig. 2). In $\Delta psbJ$ mutants the content of PSII was likewise much less than in the controls, the wild-type and RV plants. Photosystem I, LHCII trimer, cytochrome b_6f and ATP synthase were present at similar levels in all lines, independently of the mutations in the *psbEFLJ* operon.

Information about structural assembly of PSII and the identity of associated proteins was obtained from two-dimensional gel analysis (BN-PAGE followed by SDS-PAGE) combined with mass spectrometry and immunoblotting with antisera against various PSII proteins. In wild-type plants, both intact and CP43-less monomers were detected as well as the dimeric PSII and a small amount of PSII supercomplexes (Fig. 3). The cytochrome b_6f complex was found to be present as a dimer and LHCII complex as a trimer and monomer. Information about the assembly stages of PSII complexes in various *psbEFLJ* operon mutants was mainly based on immunoblotting after 2D-BN-PAGE
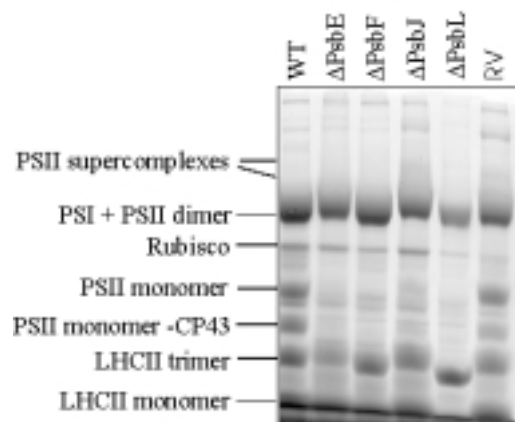
Fig. 2. Blue-native gel electrophoresis (BN-PAGE) of thylakoid protein complexes from tobacco *psbEFLJ* operon mutants and controls (WT and (RV). The protein complexes were solubilized with 1 % n-dodecyl-maltoside. 40 µg of chlorophyll was loaded in each well.

Fig. 3. Two-dimensional gel analysis of protein complexes of wild-type tobacco thylakoid membranes. The protein complexes were solubilized with 1% n-dodecylmaltoside. 40 μg of chlorophyll was loaded onto the BN-PAGE. After a run, a lane of BN-PAGE was cut, solubilized and placed horizontally on the top of the SDS-PAGE gel. The gel was silverstained. Protein spots were identified with MALDI-TOF. Similar gels were also blotted to PVDF membrane and probed with antisera against different PSII proteins. Immunoblots with anti-D1 and anti-PsbE are shown below as examples.

Discussion

We have characterized *psbEFLJ* operon mutants of tobacco with special interest on the roles of corresponding proteins for the assembly and stability of PSII complexes. The essential role of both subunits of Cyt b_{559} (PsbE and PsbF) in any stable assembly of PSII was confirmed in our studies for higher plants, similar to the situation earlier published for cyanobacteria and *Chlamydomonas* (see Introduction). Similarly, one-dimensional BN-PAGE of Δ*psbL* did not reveal any clear protein complexes in the region where PSII complexes of WT migrated indicating that PsbL is also essential for stable PSII assembly. Closer analysis of these mutants on 2D gels followed by immunoblotting, however, revealed the particular need of PsbL for stable assembly of CP43 in PSII monomer (data not shown). Δ*psbJ* plants were capable of assembling PSII dimers but not supercomplexes with associated LHCII. Also, the amount of PSII complexes was significantly lower compared to the controls, indicating that PsbJ has a role in stabilization of PSII. Our results are in line with those obtained with *Synechocystis psbJ* deletion mutant having approximately half of the PSII activity compared to the wild-type cells (Lind et al., 1993).

Synthesis and assembly of other thylakoid protein complexes occurred independently of the mutations in the *psbEFLJ* operon and thus also independently of the presence of PSII complexes in the thylakoid membrane. The presence of LHCII trimer and monomer, PSI complex, the Cyt b_6f monomer and dimer complexes as well as ATP synthase were not affected by changes in PSII assembly and function. Thus, it seems that the synthesis and assembly of the major protein complexes of the thylakoid membranes occur quite independently, without coordinated signals from other complexes.

   Absence of PSII complexes in Δ*psbE* and Δ*psbF* mutants was reflected in the chloroplast ultrastructure. Chloroplasts were round-shaped and the amount of grana stacks in chloroplasts as well as the number of thylakoid membranes per granum were low. Disturbances in grana formation in these mutants yet containing LHCII complexes at relatively high amounts, demonstrate the significance of PSII complexes to grana stacking. In Δ*psbJ* and Δ*psbL* the shape of chloroplasts as well as grana stacking resembled more of that of the wild-type plants. However, the amount of grana stacks was slightly reduced.

   We conclude that in higher plants, both subunits of Cyt b_{559} as well as PsbL protein are essential for stable assembly of PSII monomer. PsbJ, although not essential for normal assembly of PSII dimers, is required for proper function and/or stabilization of the PSII complex in thylakoid membranes.

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