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# Isolation and characterization of PSII core complexes from a brown alga, *Laminaria saccharina*

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PSII-enriched particles, active for DCIP-reduction, were prepared from *Laminaria saccharina* chloroplasts, and PSII core complexes were further purified by ion-exchange chromatography. They contained several polypeptides, four of them cross-reacting with antibodies raised against CP47, CP43, D1 and D2 of green plants. A second chromatography was required to separate: (i) a core antenna, composed of 51 kDa polypeptide subunits, binding 11 β-carotene, 4 chlorophyll (Chl) *c* and 7 fucoxanthin for 100 Chl *a*, and reacting with CP47 antibodies; and (ii) a reaction center complex consisting of two main polypeptides of 34 and 36 kDa. The pigment stoichiometry was of 5 Chl *a* and 0.5 β-carotene for 2 pheophytin *a*. The 34 and 36 kDa components cross-reacted with anti-D1 and anti-D2 antibodies, respectively. The presence of cytochrome *b*-559 was substantiated by spectrophotometry.

Brown alga; Chromophyte; D1; PSII; Pigment–protein complex

## 1. INTRODUCTION

It is currently admitted that the chloroplasts of eukaryotic organisms originated from prokaryote autotrophs (probably cyanobacteria) by endosymbiosis. Furthermore, recent phylogenetic studies, especially by means of RNA sequencing [1,2], support the hypothesis that the chloroplasts of the three main phyla of eukaryotic autotrophs (Chlorophyta, Rhodophyta and Chromophyta) are derived from distinct symbiotic events. According to this hypothesis, the question is open as to the more or less structural homology between the chloroplast pigment–protein complexes from these three groups of organisms, not only concerning the light-harvesting complexes, which are obviously rather different [3], but also the core PS complexes, which are, up to now, poorly investigated.

The PSII core complex of green plants and algae has been extensively studied, and is it now well established that its protein moiety consists of four main polypeptide subunits of 47, 43, 34 and 32 kDa (in a stoichiometry currently admitted to be 1, 1, 1, 1) accompanied by several polypeptides of lower molecular weight (MW) (for review, see [4]). The chloroplastic genes encoding the four main polypeptides and some of the smaller ones have been isolated and sequenced in several species, demonstrating a strong homology between the two 30 kDa-range subunits (D1 and D2) and the L and M subunits of the bacterial reaction center [5–7]. Photophysical studies of highly refined preparations allowed Nanba and Satoh [8] to accurately localize the primary actors of charge separation, i.e. P680 and Pheo on the D1–D2 complex, however, the stoichiometry of the pigmend and electron carrier molecules actually linked to the D1–D2 particles is still controversial: it was estimated from 3–5 chlorophyll (Chl) *a*, 1–2 β-carotene, 1–1.5 cytochrome (cyt) *b*-559 for 2 pheophytin *a* molecules, according to the various materials and isolation procedures used in the different studies [9–15].

Purified preparations of PSII core complexes have also been obtained from cyanobacteria and revealed a composition similar to the green plant ones, although the MW of the main components are not exactly the same [16]. Sequencing also demonstrated some divergence with green plants [17].

By contrast, the internal organization of PSII core from Chromophyta has not yet been investigated. Some subchloroplast fractions have been presented as PSII-enriched fractions [18] mainly according to their location on sucrose gradient or gel electrophoresis, and to their spectral characteristics. Some years ago [19], using digitonin as a mild solubilizing agent, we isolated from a brown alga, *Fucus serratus*, a 'native' fraction performing PSII activity, and determined its spectral characteristics. In the present work, using another brown alga species, *Laminaria saccharina*, we investigated the

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*Abbreviations:* bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1.3-diol; Chl, chlorophyll; CP43, CP47, D1, D2, polypeptides components of green plant PSII complexes; DCIP, 2,6-dichlorophenolindophenol; DEAE, diethylaminoethylcellulose; DPC, 2.2′-diphenylcarbazide; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; LiDS, lithium dodecyl sulphate; PS, photosystem; TCA, trichloroacetic acid.


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pigment and polypeptide composition of this fraction, and examined its immunological relationships with components of green plant PSII cores. In addition, we purified the fraction further to obtain D1–D2–cyt *b*-559 particles, the spectroscopic characteristics and pigment composition of which were determined.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

*Laminaria saccharina* (L.) Lamouroux (*Phaeophyceae*) thalli were collected near the 'Station biologique' at Roscoff (France). Chloroplasts were extracted according to [20] and resuspended in 0.01 M HEPES-Na buffer at a Chl *a* concentration of 200 μg/ml. Digitonin was added at a detergent/Chl *a* ratio of 100 just before the homogenate was passed through a French pressure cell and then loaded on the top of a sucrose gradient and centrifuged at 140,000 × *g* for 15 h at 4°C [21].

### 2.2. *Ion-exchange chromatography*

The PSII 'native' fraction was mixed with an equal volume of 50 mM Tris-HCl (pH 7.4), 0.05% dodecylmaltoside, 20 mM NaCl (buffer A). After a 60 min incubation with stirring in the dark at 4°C, the solubilized complex was loaded onto a DEAE-52 column. The column was extensively washed with buffer A until no more chlorophyll could be eluted. It was further washed with buffer A containing 75 mM NaCl: at this salt concentration, the LH complex remaining in the sample is eluted (fraction IE1). Another proteic fraction, IE2, is eluted with 150 mM NaCl in buffer A. This last fraction was then treated with 0.5% dodecylmaltoside and the chaotropic agent NaSCN (2 M) for 15 min at 4°C, and subsequently dialysed against 20 mM Bistris (pH 6.5), 0.05% dodecylmaltoside, 20 mM NaCl (buffer B). The dialysed material was loaded on a MonoQ column (Pharmacia FPLC system) that has been previously equilibrated with buffer B. The column was subjected to a gradient of NaCl from 20 mM to 500 mM.

### 2.3. *Electrophoresis*

Electrophoresis was performed in the presence of LiDS on a 12% polyacrylamide gel or in presence of SDS on a 9–18% linear polyacrylamide gradient and double Tris concentration as described in [22]. Resolving gels contained 6 M urea to increase the focusing of D1 and D2 bands. Samples were concentrated 10-fold using centricon concentrators (Amicon) or precipitation with 20% TCA.

Rabbit polyclonal antibodies against D1 polypeptide were a kind gift of Dr Ohad and those against D2 and CP47 of Dr. Friso. Antibodies against *Chlamydomonas* CP43 came from Dr Chua. Proteins were separated by acrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The blot was incubated sequentially with antiserum and peroxydase-conjugated goat anti-rabbit IgG (Bio-Rad). The peroxidase-containing bands were developed by incubating the strips with hydrogen peroxyde and 4-chloro-1-napthol.

### 2.4. *HPLC analysis and quantification of pigments*

This was performed according to [23]. The mM extinction coefficient for pheophytin *a* was 131 at 409 nm in 85% acetone.

### 2.5. *Detection of cyt* b-*559*

Detection of cyt *b*-559 (reduced versus oxidized absorption difference at 559 nm) was performed immediately after the preparation of the complexes using an Aminco spectrophotometer [24].

## 3. RESULTS AND DISCUSSION

### 3.1. *Isolation of 'native' PSII-enriched particles on density gradient*

As already described for *Fucus serratus* in [21], and as shown on the 680 nm absorbance profile (Fig. 1A), several discrete pigmented layers were usually observed on sucrose density gradient after solubilization of membranes by digitonin. The lighter a wide orange-brown band, was the previously characterized LH complex [25]. Two green, heavy bands were PSI-enriched particles [23]. Another less abundant green band, located at 20% sucrose, has already been proved in *Fucus* [19] to

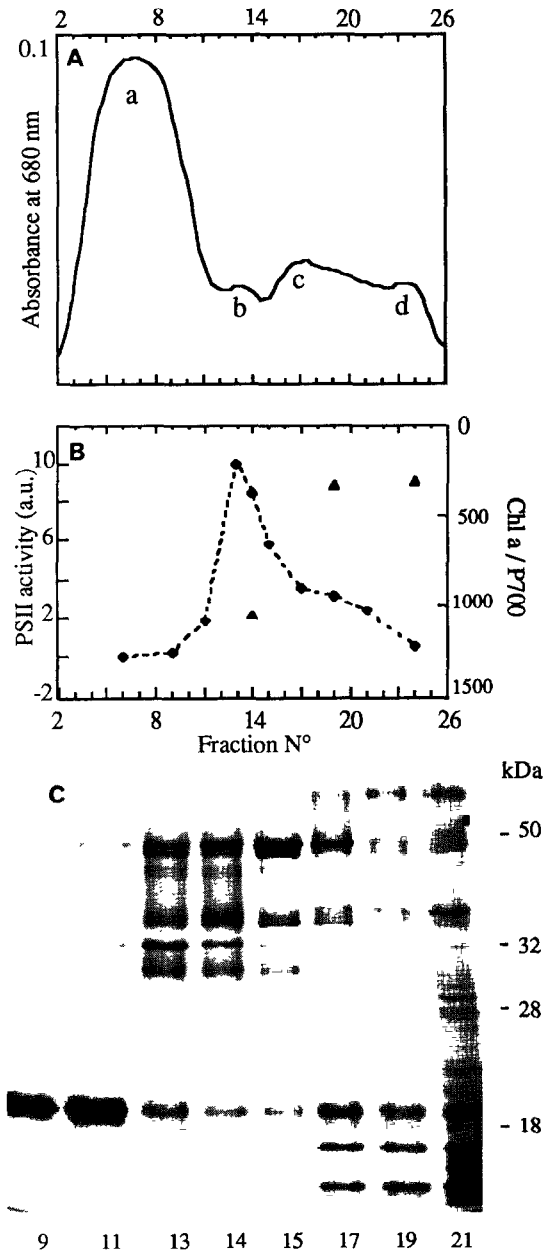


Fig. 1. Separation, on a sucrose density gradient, of pigment–protein complexes from digitonin-treated chloroplasts. (A) Absorbance profile at 680 nm: a, LH complexes; b, 'native' PSII particles; c and d, native PSI particles. (B) Rate of DCIP reduction in the presence of DPC (●) and Chl *a*/P700 ratios (▲) obtained from successive layers of the gradient. The measurements were performed with an Aminco double beam spectrophotometer as described in [20] and [23]. (C) Acrylamide gel electrophoresis (Coomassie blue stained) of aliquots from the successive layers of the gradient.

contain PSII centers: although it was not oxygen-evolving, it was able to reduce DCIP in presence of DPC [19]. In the present work, the DPC-to-DCIP electron flux was evaluated for the successive fractions of the gradient (Fig. 1B). This PSII activity (relative to Chl *a* concentration) was maximum in the narrow discrete green band (fractions 12–14), however, it was not entirely restricted to it, but present also in the lower fractions. Conversely, fractions 12–14 were depleted in P700.

The polypeptide pattern of fractions 9–21 is presented in Fig. 1C. Polypeptides with MW between 30 and 50 kDa were dominant in the PSII-enriched zone, but several other polypeptides were also present, especially a 20 kDa one which is the major polypeptide of the LH complex as seen in lane 9.

The PSII 'native' particles (fraction 13) exhibited a maximum of fluorescence at 687 nm at room temperature, and also at 77 K (data not shown), at a longer wavelength than the LH complex (fractions 6–9) which fluoresces at 677 nm. Its pigment composition is given in Table I. The specific bands of Chl *c* and carotenoids are low, but still present in the absorption spectrum (Fig. 2A). The fluorescence excitation spectrum (Fig. 2B) showed that at least part of the Chl *c* and xanthophylls present in the particles are still connected to Chl *a*. This, as well as the polypeptide profile, demonstrated that these native PSII particles were not yet pure PSII core complexes, and required further purification.

### 3.2. *Purification of the PSII core*

In view of recovering with a good yield the PSII centers (present at a rather low concentration in the chloroplast extract due to the large antenna size in this alga), fractions 11–14 have been pooled and loaded on a DEAE column. The first fraction, IE1, was eluted with the 75 mM NaCl buffer. It presented the characteristics of the LH complex, i.e. it was enriched in Chl *c* and xanthophylls (Table I and Fig. 2A), and composed of 20 kDa polypeptide units (Fig. 3, lane 3). This 20 kDa polypeptide was recognized (Fig. 4, lane 1) by antibodies raised against the LH polypeptide from *Fucus* LH [26]. It fluoresced mainly at 677 nm (data not shown) and the fluorescence excitation spectrum (Fig. 2B)

Table I

Pigment composition of isolated fractions

| Fraction | Native[a] | IE1[a] | IE2[a] | IE2[b] | M1[a] | M2[a] | M2[b] |
|---|---|---|---|---|---|---|---|
| Phenophytin *a* | 3.3 | 0.8 | 3.3 | 2 | 0.4 | 38.7 | 2 |
| Chlorophyll *a* | 100.0 | 100.0 | 100.0 | 34 | 100.0 | 100.0 | 5.1 |
| *β*-Carotene | 13.9 | 4.7 | 16.0 | 7 | 11.1 | 7.9 | 0.4 |
| Chlorophyll *c* | 7.1 | 20.7 | 3.0 | 1 | 3.6 | 3.1 | 0.1 |
| Fucoxanthin | 25.8 | 90.1 | 9.5 | 2 | 6.8 | 1.7 | <0.1 |
| Violaxanthin | 4.7 | 1.4 | 3.1 | <1 | n.d. | 0 1 | <0.1 |

The results are expressed in molar concentrations relative to 100 Chl *a*[a] or to 2 pheophytin *a*[b].

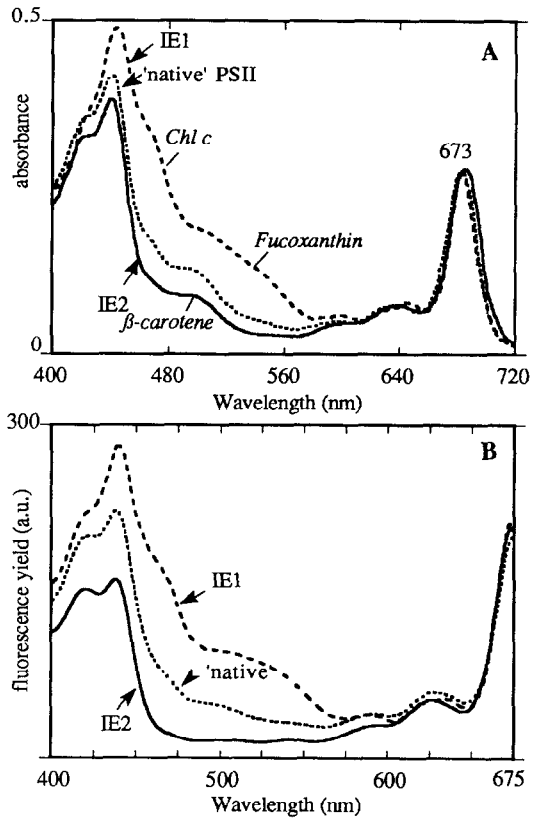


Fig. 2 Absorption spectra (A) and fluorescence excitation spectra (B) at room temperature of the 'native' PSII particles (dotted line) of the LH complex (IE1, dashed line) and of the PSII core (IE2, solid line). Slit width for excitation and emission was 3 nm. Emission was recorded at 687 nm.

showed that the fucoxanthin was still energetically connected to Chl *a*.

The fraction eluted with 150 mM NaCl, IE2, still presented some DCIP reduction activity, and as such it could be considered a PSII core complex. Its spectral characteristics, pigment composition and polypeptide profile are given in Table I and Figs. 2 and 3. Compared to the PSII native particles, it was highly depleted in Chl *c* and xanthophylls, which have been recovered with the IE1 peak, and was enriched in *β*-carotene (Table I), which was clearly seen in the absorption spectrum (Fig. 2A). By contrast, the absence of the *β*-carotene band in the excitation spectrum (Fig. 2B) indicated that this pigment did not transfer energy to Chl *a*. The pigment stoichiometry of IE2 (34 Chl *a* and 7 *β*-carotene for 2 pheophytin *a*) is in agreement with that reported from PSII core particles of green plants [10]. The fraction revealed several polypeptides in the 30–50 kDa range. In order to identify them, Western blotting using antibodies raised against PSII polypeptides from green organisms were carried out. The 34 kDa band reacted with the anti-D1 antibodies, the 36 kDa band with the anti-D2 antibodies, while reactions with the antibodies raised against the CP43 of *Chlamydomonas* and against

the CP47 were observed in the 50 kDa range (three of these immunoreactions are shown in Fig. 4). A polypeptide with an apparent MW of 12 kDa was presumably representing cyt *b*-559. Thus four major polypeptides of this complex shared some homology with the four polypeptides of green organism PSII. We can infer that it is comparable to the PSII core particles described from green plants [8–15], green algae [27] and cyanobacteria [16]. In a recent work [28], Plumley et al. noticed cross-reactivity of thylakoids from a diatom with antibodies raised against *Chlamydomonas* PSII polypeptides.

This PSII core complex was further dissociated on an ion-exchange (MonoQ) column into two main fractions.

(1) The first fraction, M1, eluted at 200 mM NaCl, and contained a Chl *a* binding polypeptide of an apparent MW of 51 kDa (Fig. 3, lane 4). Its red absorption maximum was at 673 nm (Fig. 5A). Its 77 K fluorescence emission maximum was at 682 nm at room temperature and 685 nm at 77 K; it contained very little pheophytin *a* but, as in IE2, some Chl *c* and fucoxanthin were observed (Table I). It cross-reacted with the 47 kDa antibodies (not shown).

(2) From fraction M2, eluted at 300 mM NaCl, two polypeptides with apparent MW of 34 and 36 kDa were resolved by 6 M urea electrophoresis (Fig. 3, lane 6). The anti-D1 antibodies cross-reacted with the 34 kDa polypeptide (Fig. 4, lane 3), the anti-D2 with the 36 kDa (Fig, 4, lane 5). Both recognized a higher molecular aggregate of D1–D2 dimers present in some preparations, mainly when fractions had been precipitated with TCA (Fig. 4, lanes 5 and 6). Similar results have already

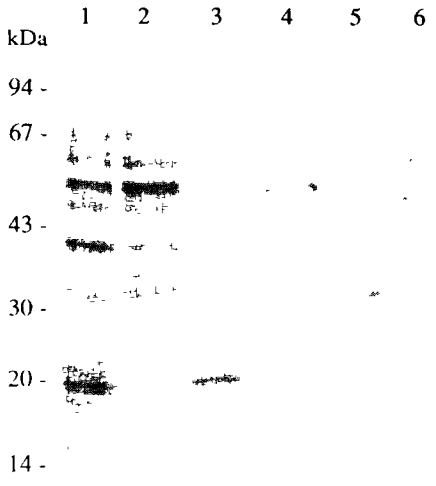


Fig. 3. Electrophoresis (Coomassie blue stained) on a 9–18% linear polyacrylamide gradient, 6 M urea, double Tris gel. Lane 1, 'native' PSII-particles; lane 2, PSII core (IE2); lane 3, LH complex (IE1); lane 4, PSII core antenna (M1); lanes 5 and 6, two types of PSII reaction center (M2) obtained. On lane 5, a residual band at 51 kDa is observed; on lane 6, only 34 and 36 kDa polypeptides are present. Positions of MW markers are shown in the left lane.

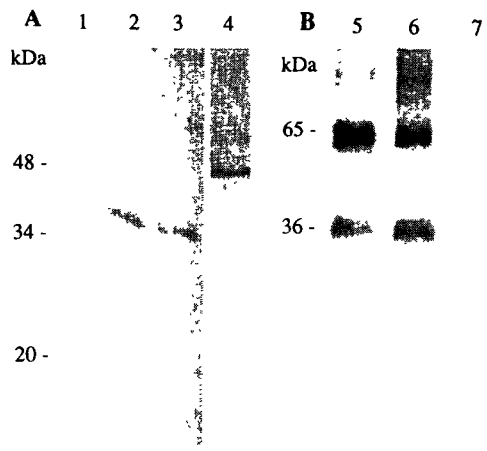


Fig. 4. Immunoblotting using antibodies against: the LH on IE1 fraction (lane 1); D1 on IE2 (lane 2) and M2 (lane 3) fractions; CP43 on IE2 (lane 4) fraction; D2 on M2 (lane 5), IE2 (lane 6) and 'native' PSII (lane 7) fractions.

been observed in higher plants and cyanobacteria [8,9,16]. No reaction with the anti-43 kDa antibodies was obtained with these fractions. It is possible that the positively reacting polypeptide, present in the PSII core complex, IE2, was lost during the separation procedure. In some cases (Fig. 3, lane 5) the fraction still contained a small amount of the 51 kDa polypeptide, as observed in the p5 fraction in Ghanokatis et al. [11].

In most cases, the electrophoresis system used did not resolve the cyt *b*-559 polypeptides in our preparations. Spectrophotometrically [24], a difference in absorbance between oxidised (ammonium persulfate) and reduced (dithionite) samples was observed in the 559 nm range, but, due to the very low concentration of the M2 fraction, the quantification of this component was not accurate.

From the polypeptide MW and the immunological cross-reactivity, we can infer that M1 originated from PSII core antenna, and that M2 was composed of PSII reaction centers, homologous to green plant D1–D2 cyt *b*-559 particles. The pigment stoichiometry observed (Table I) was in agreement with those of green plant counterparts. The Chl *a*/pheophytin *a* ratio was also consistent with green plant material. The $\beta$-carotene content was rather low, probably due to some photodestruction or loss during the isolation procedure [29]. The room temperature absorption spectrum of this fraction exhibited a red maximum at 673 nm (Fig. 5A). In the blue range, the absorption bands of pheophytin *a*, Chl *a* and $\beta$-carotene were clearly observed at 418, 438 and 494 nm, respectively. Contrary to what is currently observed in green plant D1–D2 particles, the pheophytin *a* peak in the blue range was lower than that of Chl *a*: this could be due to its lower content in cyt *b*-559 [8]. The fluorescence at room temperature was maximum at 682 nm. At 77 K, the fluorescence emission

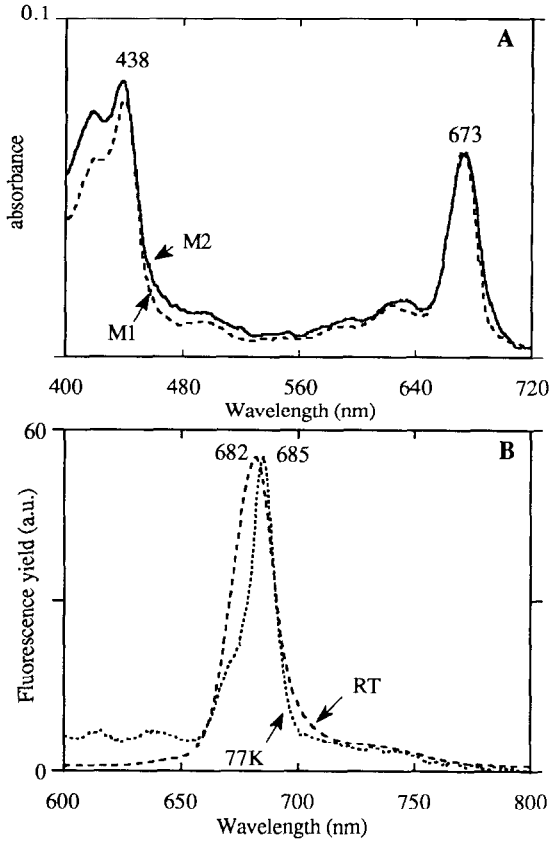


Fig. 5. (A) Absorption spectra at room temperature of the PSII core antenna (M1) and the reaction center complex (M2). (B) Fluorescence emission spectra at room temperature and at 77 K of the reaction center complex. Excitation was at 440 ± 5 nm. Emission was measured with a slit width of 3 nm. Spectra are normalized at emission maximum wavelength.

spectrum exhibited a peak at 685 nm and a shoulder near 673 nm (Fig. 5B). It was similar to the spectrum of the PSII reaction center from pea presented in [9], and suggests two discrete sources of fluorescence even in this rather simple particle.

In conclusion, PSII core and PSII reaction center complexes have been isolated from a brown alga for the first time, and their polypeptide and pigment compositions have been resolved. The results presented show that the molecular organization of the brown alga PSII core complex was similar to that of higher plants and cyanobacteria, however, their polypeptide components have MW somewhat higher than green plants and more similar to cyanobacteria. Up until now, only one gene specifying for a polypeptide of the brown alga PSII reaction center has been sequenced [30]; this gene, psbA, encoding the D1 polypeptide, is highly homologous with that of green plant, although with some differences, especially the presence near the carboxy-terminus of a seven amino acid insertion found in cyanobacteria and a red alga.

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