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Isolation of a *psaF*-deficient mutant of *Chlamydomonas reinhardtii*: efficient interaction of plastocyanin with the photosystem I reaction center is mediated by the PsaF subunit

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The PsaF polypeptide of photosystem I (PSI) is located on the lumen side of the thylakoid membrane and its precise role is not yet fully understood. Here we describe the isolation of a *psaF*-deficient mutant of the green alga *Chlamydomonas reinhardtii* generated by co-transforming the nuclear genome of the *cw15-arg7A* strain with two plasmids: one harboring a mutated version of the *psaF* gene and the other containing the argininosuccinate lyase gene conferring arginine prototrophy. This *psaF* mutant still assembles a functional PSI complex and is capable of photoautotrophic growth. However, electron transfer from plastocyanin to $P700^+$, the oxidized reaction center chlorophyll dimer, is dramatically reduced in the mutant, indicating that the PsaF subunit plays an important role in docking plastocyanin to the PSI complex. These results contrast with those obtained previously with a cyanobacterial *psaF*$^-$, *psaJ*$^-$ double mutant where no phenotype was apparent.

Keywords: *Chlamydomonas*/nuclear transformation/photosystem I/plastocyanin docking subunit/*psaF*

Introduction

The photosystem I (PSI) complex of higher plants, algae and cyanobacteria is a light-driven plastocyanin:ferredoxin oxidoreductase consisting of at least 14 subunits (for review, see Golbeck and Bryant, 1991; Golbeck, 1992). P700, the primary electron donor of PSI, is bound to the two larger reaction center polypeptides near the luminal side of the thylakoid membrane and thus accessible to plastocyanin, the secondary electron donor. At least two small molecular weight subunits, PsaF and PsaN, are located on the luminal side of the PSI complex (Franzen *et al.*, 1989; Knoetzel and Simpson, 1993). While the role of PsaN is largely unkown, results of cross-linking experiments suggest that PsaF is involved in docking the soluble plastocyanin and cytochrome c553 polypeptides to the PSI core complex (Wynn and Malkin, 1988; Wynn *et al.*, 1989a,b). It could be shown that the cross-linked plastocyanin interacts functionally with and reduces $P700^+$ (Hippler *et al.*, 1989). The PsaF polypeptide is also associated with the peripheral light-harvesting antenna complex of PSI (LHC-I complex) in different photosynthetic organisms (Anandan *et al.*, 1989; Bassi *et al.*, 1992). The loss of the PsaF polypeptide from PSI core complexes devoid of LHC-I suggests that this subunit is more tightly bound to LHC-I than to PSI (see Wynn *et al.*, 1989a; Scheller and Moller, 1990). In *Chlamydomonas reinhardtii*, however, a mutant lacking LHC-I retains the PsaF polypeptide in the thylakoid membranes (Wollman and Bennoun, 1982).

The interactions between plastocyanin and PSI have been studied previously by measuring the kinetics of reduction of photo-oxidized P700 by flash absorption spectroscopy using various biological materials, such as solubilized PSI reaction centers and plastocyanin (Haehnel *et al.*, 1980; Bottin and Mathis, 1985), isolated chloroplasts of higher plants (Bottin and Mathis, 1987; Haehnel *et al.*, 1989) or intact green alga (Delosme, 1991). In most cases, the reduction of $P700^+$ is biphasic. It is generally agreed that the fast phase corresponds to the electron transfer from PSI-bound plastocyanin to P700, whereas the slower phase reflects a bimolecular reaction between free plastocyanin and PSI. The half-time of the fast phase for chloroplasts or solubilized preparations of PSI and plastocyanin from higher plants is 10–13 μs (Bottin and Mathis, 1987; Haehnel *et al.*, 1989) and for intact algal cells it is 4 μs (Delosme, 1991). In both chloroplasts and intact cells, >90% of $P700^+$ is re-reduced with μs kinetics following a saturating short flash, indicating that most PSI centers contain bound plastocyanin (Haehnel *et al.*, 1989; Delosme, 1991). At least two sites of plastocyanin appear to interact with PSI (Cookson *et al.*, 1980; Guss and Freeman, 1983; Sykes, 1985): one site is close to the copper ligand His87 at the 'northern' hydrophobic end and the other is close to the more remote Tyr83 at the 'eastern' acidic patch. Haehnel *et al.* (1994) suggested a two-step mechanism for the docking of plastocyanin to PSI. First, a long-range electrostatic interaction between the basic PsaF subunit and the acidic patch around Tyr83 of plastocyanin and, second, docking of plastocyanin with its flat hydrophobic surface to PSI in a conformation optimal for electron transfer to P700. Binding of plastocyanin and fast electron transfer to PSI was shown to depend on an interaction of negative charges of plastocyanin with basic residues of the PsaF subunit (Hippler *et al.*, 1990).

Directed deletion of the *psaF* gene in the cyanobacterium *Synechocystis* sp. PCC6803 did not affect the rate of P700 re-reduction by cytochrome c553, the electron donor of photosystem I (Xu *et al.*, 1994). This *psaF*$^-$ mutant grows photoautotrophically and possesses a fully active PSI complex (Chitnis *et al.*, 1991). The only alteration observed was a greater susceptibility of the PSI complex of this strain to thermolysin *in vitro* (Xu *et al.*, 1994). Similarly, removal of the PsaF subunit from the

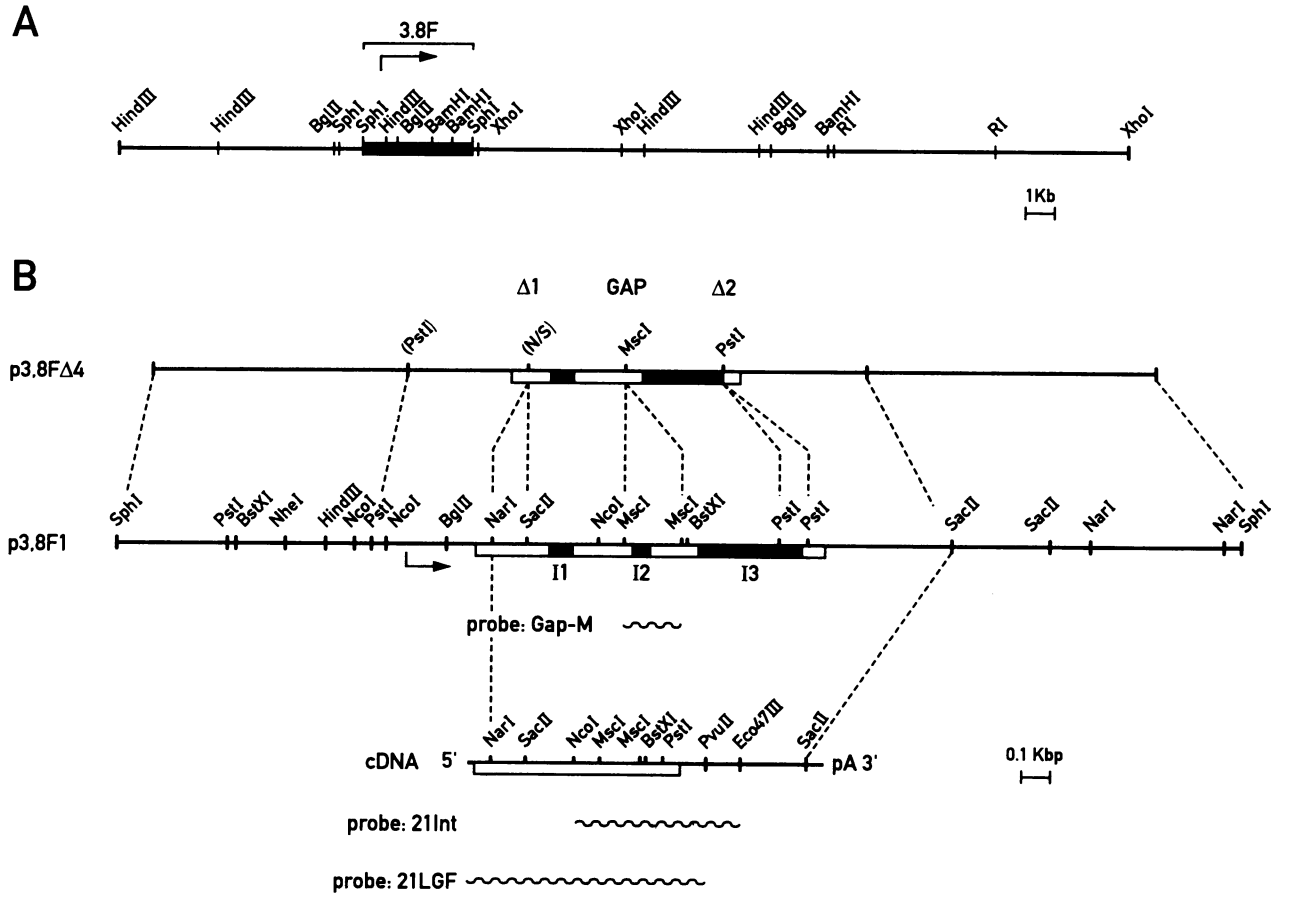

Fig. 1. (**A**) Restriction map of the p21cos16 cosmid insert, containing the *psaF* gene of *C.reinhardtii*. The insert is 35 kbp long. The *psaF* gene is located on the 3.8 kbp *Sph*I fragment (thick line: 3.8F fragment). Note that not all *Sph*I sites are shown. RI; *Eco*RI. The arrow indicates the direction of transcription of the *psaF* gene. (**B**) Restriction map of the genomic 3.8F fragment (plasmid p3.8F1). The location of the coding sequence of the *psaF* gene (open bar) on the 3.8F fragment is inferred from the restriction map of the *psaF* cDNA shown below. The difference in length of some of the restriction fragments between cDNA and genomic DNA indicates the presence of introns (black bars: I1, I2, I3). The structure of the *psaF* deletion derivative (p3.8FΔ4) is shown above the restriction map of 3.8F, with the deletions Δ1 (*Nar*I–*Sac*II), GAP (*Msc*I–*Msc*I) and Δ2 (*Pst*I–*Pst*I). The upstream *Pst*I site has been destroyed during the construction of this plasmid. The Gap-M probe corresponds to the genomic *Msc*I–*Msc*I fragment. *psaF* cDNA probes 21Int (*Nco*I–*Eco*47III) and 21LGF (*Eco*RI–*Pvu*II) are indicated; pA, poly(A) extension of the cDNA. The arrow indicates the direction of transcription of the *psaF* gene.

PSI complex of *Synechococcus elongatus* did not alter the rate of electron transfer from cytochrome c553 to P700 (Hatanaka *et al.*, 1993).

In view of these surprising results, we have isolated and characterized a *psaF*-deficient mutant of *C.reinhardtii*. We have thereby achieved the first inactivation of a nuclear gene encoding a PSI subunit in a photosynthetic eukaryote. As observed for the *psaF*$^-$ cyanobacterial mutant, the *C.reinhardtii* mutant is able to grow photoautotrophically, although its fluorescence transients are altered, as compared with wild-type, when grown in low light. The most remarkable feature of this mutant, however, is a considerable decrease in the rate of electron transfer from plastocyanin to P700$^+$, indicating that the PsaF subunit is important for this process.

Results

Isolation of the psaF gene

A restriction map of the 40 kbp p21cos16 cosmid clone containing the *psaF* gene is shown in Figure 1A. This clone was isolated from the *C.reinhardtii* cosmid genomic library P using the 21LGF probe derived from the cDNA of *psaF* (Figure 1B, Franzen *et al.*, 1989; Purton and Rochaix, 1994).

The 3.8 kbp *Sph*I fragment, 3.8F, containing the *psaF* gene was isolated and cloned, giving rise to the p3.8F1 plasmid. The location of the *psaF* gene on p3.8F1 and the corresponding restriction map are shown in Figure 1B. The presence of introns was inferred from the difference in size between some of the corresponding restriction fragments of the *psaF* gene and its cDNA. This was confirmed for the genomic *Msc*I–*Msc*I restriction fragment from which the 62 bp intron I2 was sequenced (not shown).

Inactivation of the psaF gene in C.reinhardtii

This work was started with the assumption that the PsaF subunit is essential for the reduction of P700$^+$ by plastocyanin or by cytochrome c553 (Bengis and Nelson, 1977; Wynn and Malkin, 1988; Hippler *et al.*, 1989; Wynn *et al.*, 1989a,b) and that inactivation of *psaF* would lead to a PSI-deficient phenotype. The initial strategy was therefore to inactivate *psaF* of *C.reinhardtii* through replacement with a mutant gene copy by transformation

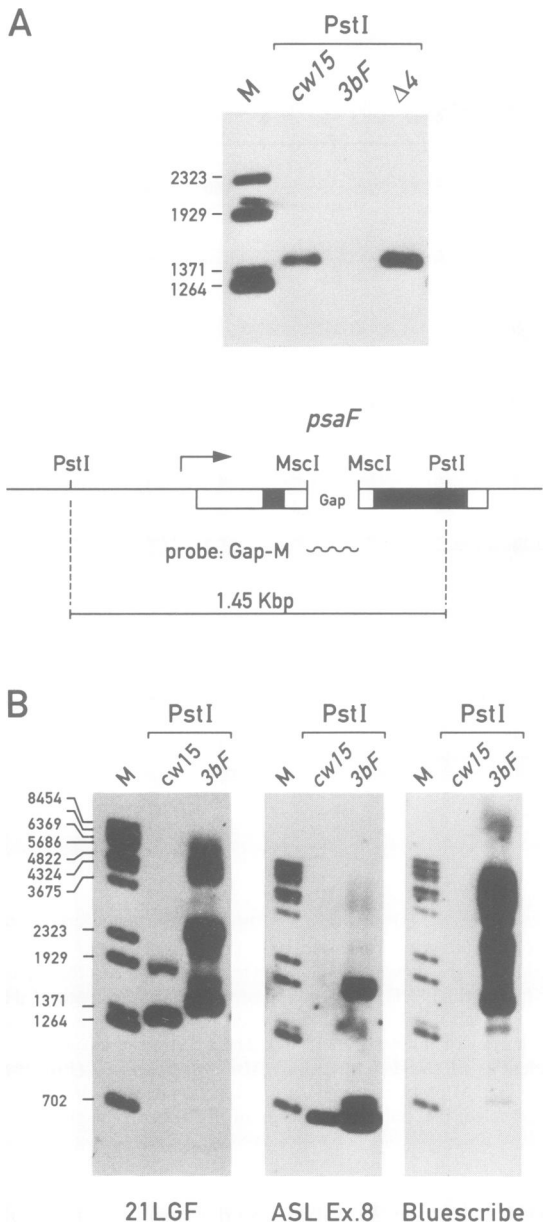

Fig. 2. Southern blot analysis of the 3bF mutant. (**A**) *Pst*I digests of total DNA from the indicated *C.reinhardtii* strains were hybridized with the Gap-M probe. Δ4 is a PSI-deficient mutant isolated during this study. Lane M includes labeled *Bst*EII-digested λDNA used as size marker (values are in bp). The structure of the *psaF* locus with the position of the probe and the size of the expected fragment after *Pst*I digestion in a wild-type strain is shown below. The arrow indicates the direction of transcription. (**B**) *Pst*I digests of total DNA isolated from cw15 and 3bF hybridized with the probes 21LGF, ASL Ex.8 (180 bp fragment derived from exon 8 of the argininosuccinate lyase gene) and Bluescribe plasmid. The same filter was re-used for the three hybridizations after stripping off the previous probe.

and homologous nuclear recombination and to screen the transformants by fluorescence transients. For this purpose, we created three small deletions in *psaF* (labeled Δ1, Gap and Δ2 in Figure 1B) to generate the plasmid p3.8FΔ4 (for details see Materials and methods).

Prior to transformation, the p3.8FΔ4 plasmid was linearized with *Msc*I which created a gap and provided DNA ends which can stimulate homologous recombination (Orr-Weaver *et al.*, 1981; Kucherlapati *et al.*, 1984; Sodeinde and Kindle, 1993). The linearized p3.8FΔ4 and the closed circular pArg7.8 plasmid, containing the argininosuccinate lyase (ASL) gene of *C.reinhardtii* (Debuchy *et al.*, 1989) were introduced in the cw15-arg7A strain by co-transformation and plated on TAP medium lacking arginine. The frequency of nuclear co-transformation with these two plasmids was 60% or more, as reported previously by Kindle (1990). The recovered arg^+ transformants were screened by fluorescence transients for a block in electron transfer between the plastoquinone pool and PSI (Bennoun and Delepelaire, 1982). Among 1.6×10^9 plated cells, 22 000 had an arg^+ phenotype and 110 were affected in photosynthetic electron transfer. The DNA of these mutants was tested for alterations at the *psaF* locus. This screening revealed one mutant, called 3bF, whose DNA upon *Pst*I digestion did not hybridize with the Gap-M probe (Figure 2A) corresponding to the gap in the transforming DNA (Figure 1). Hybridization of *Pst*I-digested DNA of 3bF with the 21LGF probe (containing more than half of the *psaF* cDNA, Figure 1B), revealed the presence of multiple insertions of the mutated *psaF* gene and confirmed the absence of the wild-type 1.45 kbp band (Figure 2B). Hybridization with the ASL probe also revealed several insertions of the ASL gene in the nuclear genome of 3bF (Figure 2B). Finally, hybridization with the pBS(+) vector showed that it is present in several copies in the nuclear DNA of 3bF.

Genetic crosses of 3bF with wild-type revealed that not all the inserted copies of p3.8FΔ4 are tightly linked since some segregated in the $psaF^+$progeny (not shown). The results differ from earlier nuclear transformation studies where all transforming plasmid copies were linked (Kindle *et al.*, 1989). To confirm that the *psaF* gene is not expressed in the 3bF mutant, RNA was prepared from the $psaF^+$ cw15 strain and from 3bF and hybridized with a specific *psaF* probe. Figure 3A shows that *psaF* RNA is undetectable in 3bF. However, the mutant accumulates normal levels of *psaE* RNA encoding another PSI subunit. Immunoblot analysis of proteins from wild-type, the PSI mutant Δ4 and 3bF probed with antibodies against the PsaF subunit further confirmed the absence of this protein in 3bF (Figure 3B). This polypeptide is also missing from the PSI mutant Δ4, as previously reported for PSI-deficient strains (Girard *et al.* 1980).

Rescue of the 3bF mutant

Attempts to map accurately the deletion at the *psaF* locus in the mutant 3bF were unsuccessful because of the integration of several copies of the modified *psaF* gene in the genome (Figure 2B) and because of the presence of numerous repetitive sequences in the *psaF* region (data not shown). The size of the deletion was estimated to be at least 16 kbp, based on several Southern hybridizations (data not shown). To rule out the possibility that the observed fluorescence phenotype of 3bF was due to the inactivation or deletion of another gene in the vicinity of *psaF*, a wild-type copy of the *psaF* gene was tested for the ability to rescue the 3bF phenotype by co-transformation, using *nit1*, which encodes nitrate reductase, as selectable marker. Since 3bF is $nit1^-$ and $nit2^-$, it was transformed with the plasmid p3.8 F1 containing wild-type *psaF* together with the plasmids pMN24 and pMN68 encoding the wild-type *nit1* and *nit2* genes,

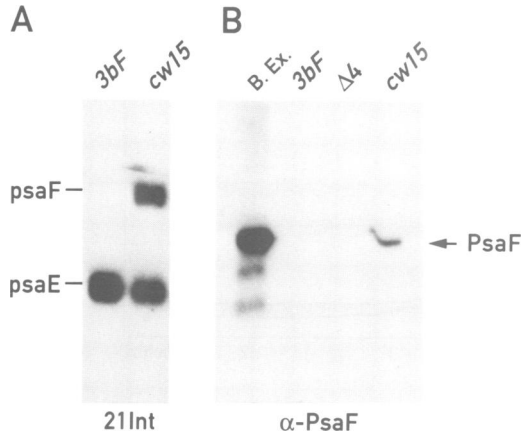

Fig. 3. The *psaF* product is not expressed in the 3bF mutant. (**A**) Northern analysis. Total RNAs (6 μg per lane) from 3bF and from cw15 were fractionated on a 1.2% agarose–formaldehyde gel and blotted. The probes used were: 21Int (Figure 1B) and a 440 bp fragment derived from the *psaE* cDNA. The positions of the *psaF* and *psaE* transcripts are indicated on the left. (**B**) Western analysis. Total cell extracts from the indicated *C.reinhardtii* strains and from an *E.coli* strain overexpressing the recombinant PsaF subunit (see Materials and methods) were fractionated on a 15% polyacrylamide gel and blotted onto a nitrocellulose membrane. B. Ex., bacterially expressed PsaF protein. The membrane was incubated with purified anti-PsaF antibodies and the immune complexes were detected with [^{125}I]protein A.

respectively, as described in Materials and methods. Three *nit*$^+$ transformants were recovered from 9×10^7 cells plated on selective medium with nitrate as sole nitrogen source. Two of these transformants had wild-type fluorescence transients indistinguishable from the cw15-arg7A strain. DNA analysis of one of these transformants, ResF2, revealed that the 3.8 kb insert of p3.8F1 is present in intact form in this strain (data not shown). Immunoblot analysis of ResF2 total cell extracts showed that the PsaF polypeptide accumulates as in wild-type in this rescued strain (data not shown). It can therefore be concluded that the ResF2 strain has acquired a functional copy of the *psaF* gene.

Analysis of the psaF-deficient 3bF mutant

The 3bF mutant is capable of growing photoautotrophically in high light (86 mE/m^2/s) nearly at the same rate as wild-type (data not shown). Although the fluorescence transients of young mutant and wild-type cultures are similar, there are clear differences in the fluorescence patterns when the cells are grown under low light (0.4 mE/m^2/s) for 2 days or more (Figure 4A). After reaching its maximal intensity, the fluorescence of 3bF no longer decreases, which is typical of PSI- or cyt b6/f-deficient mutants. In contrast, in cells from wild-type and ResF2, the fluorescence intensity rises and then declines. It is noteworthy that the changes in fluorescence transients observed with 3bF occur in cells that are still in exponential growth (Figure 4A and B). These differences in fluorescence pattern were used for the screening of mutants and allowed us to isolate 3bF. The amount of PSI complex estimated by immunoblot analysis using antibodies against the PsaD and PsaE subunits was found to be slightly reduced in 3bF relative to wild-type and ResF2 (data not shown).

The rate of electron transfer from plastocyanin to P700 is reduced in the absence of the PsaF polypeptide

To test whether electron transfer between plastocyanin and P700$^+$ is altered in the 3bF mutant, cells from wild-type, ResF2 and 3bF were illuminated with Xenon flashes of increasing intensity and the total number of PSI charge separations was measured as described in Materials and methods. The duration of the Xenon flash used (~20 μs including the tail) exceeds the turnover time of the PSI reaction center, which is mainly determined by the rate of reduction of P700$^+$ whose half-time is 4 μs in algal cells (Delosme, 1991). As shown previously, double charge separations are expected under these conditions (Joliot and Delosme, 1974). To calibrate the response, cells were illuminated with a saturating ruby laser flash with a duration of <100 ns, much shorter than the turnover time of the PSI reaction center. Under these conditions, all PSI centers undergo a single charge separation. It can be seen in Figure 5 that, in the wild-type and ResF strain, the Xenon flash of the highest energy induces a larger number of charge separations than a laser flash of equal energy. In contrast, in mutant 3bF, the number of charge separations induced by the laser and the Xenon flash are identical, indicating that the Xenon flash does not induce double hits. This shows that the rate of P700$^+$ reduction is considerably diminished in the 3bF mutant as compared with the wild-type, and suggests that the interaction between plastocyanin and P700 is altered. The restoration of the wild-type phenotype in the ResF2 strain indicates that the diminished rate of reduction of P700$^+$ in the 3bF mutant is associated with loss of the PsaF polypeptide.

Figure 5 also shows that the initial slope of the saturation curves is identical for the three strains, indicating that the mutation in 3bF does not induce significant changes in the PSI antenna size or in the efficiency of excitation transfer between the antenna and the PSI centers. The antenna appears to be essentially homogenous, based on the fact that the saturation curve can be fitted with an exponential function.

Because of the fast reoxidation of the PSI acceptors, the recovery of the photoactive PSI reaction centers is limited by the rate of reduction of P700$^+$ (Thurnauer and Norris, 1980; Thurnauer *et al.*, 1982). Figure 6 shows the kinetics of this recovery after illumination by a saturating laser flash. The amount of PSI reaction centers with reduced P700 was measured by firing a second oversaturating Xenon flash at variable time intervals after the laser flash. In Figure 6, the number of charge separations induced by the second flash is plotted as a function of time. About 100% of P700$^+$ is re-reduced in <16 μs in wild-type. This fast phase, which is missing in the 3bF mutant, can be ascribed to the electron transfer reaction occurring within the plastocyanin–PSI complex:

$$\text{PC–P700} \xrightarrow{(1)} \text{PC–P700}^+ \xrightarrow{(2)} \text{PC}^+\text{–P700}$$

The amplitude of this fast phase is equal to the number of charge separations induced by the saturating laser flash which shows that ~100% of the PSI centers are bound to plastocyanin. This first recovery phase is followed by a second phase with a half-time of 60 μs. Taking into account that the second flash is able to induce double

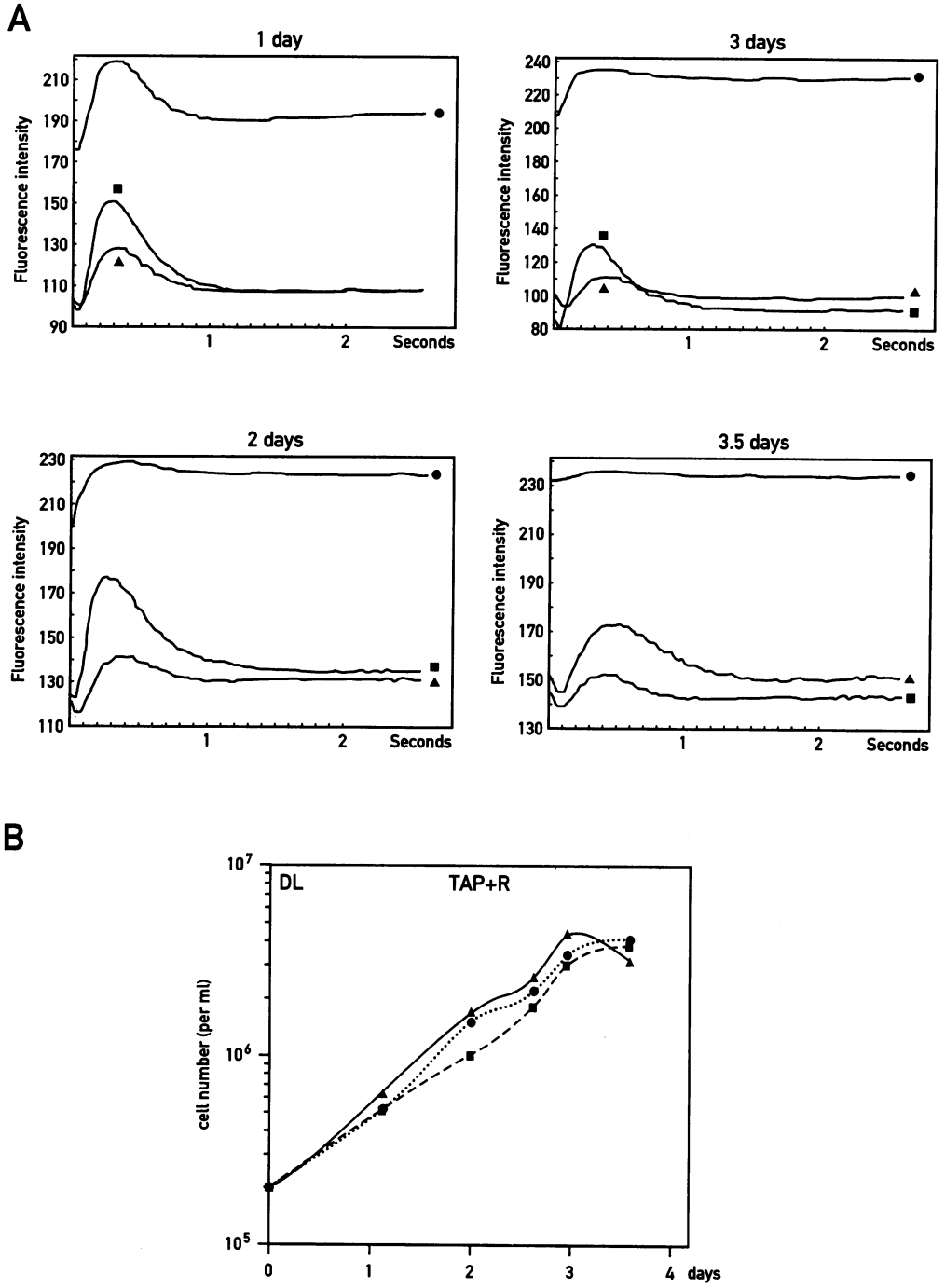

Fig. 4. (**A**) Fluorescence transients of the cw15-arg7A (▲), ResF2 (■), 3bF (●) strains, grown as liquid cultures incubated in dim light (DL, 0.4 μE/m^2/s). Cells were inoculated at a concentration of 2×10^5/ml in TAP medium supplemented with arginine. Samples were withdrawn after 1, 2, 3 and 3.5 days, and their fluorescence transients determined. Fluorescence intensities are in arbitrary units. (**B**) Growth curves of the cw15-arg7A (▲); ResF2 (■); 3bF (●) strains. The cell concentration of the samples used in Figure 5A was determined and plotted on a semi-logarithmic scale.

photoreactions, the second phase can be associated with reactions leading to the recovery of the PC–P700 complex.

$$\text{PC}^+\text{–P700} \xrightarrow{(3)} \text{PC}^+ + \text{P700}$$

$$\text{PC} + \text{P700}^+ \xrightarrow{(4)} \text{PC–P700}^+$$

The recovery phase of active photocenters in the 3bF mutant is considerably slower than in wild-type (Figure 7). It is exponential with a half-time of 1.2 ms, which corresponds to an apparent first order rate constant of 600 s^{-1}. This phase depends on both reactions (3) and (4). If one assumes that the latter is rate-limiting, this would imply that the rate of binding of plastocyanin to PSI is 20 times slower in 3bF as compared with wild-type. The absence of double hits in the mutant indicates that the complex between plastocyanin and PSI, which leads to fast electron transfer, does not form either because of a lower affinity of reduced plastocyanin for PSI or because docking does not occur properly.

The oxidation of cytochrome f is initiated by a transfer

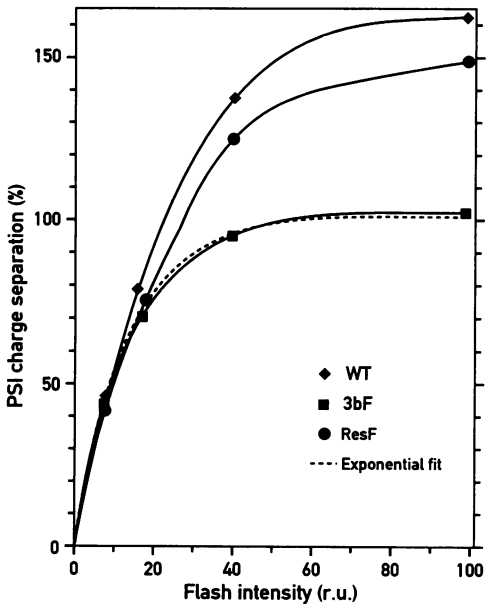

Fig. 5. Saturation curves of PSI charge separation induced by an actinic Xenon flash of increasing energy in the WT (◆), ResF (●) and 3bF (■). The data have been normalized to the signal induced by a saturating laser flash taken as 100% of PSI charge separation. r.u, relative units.

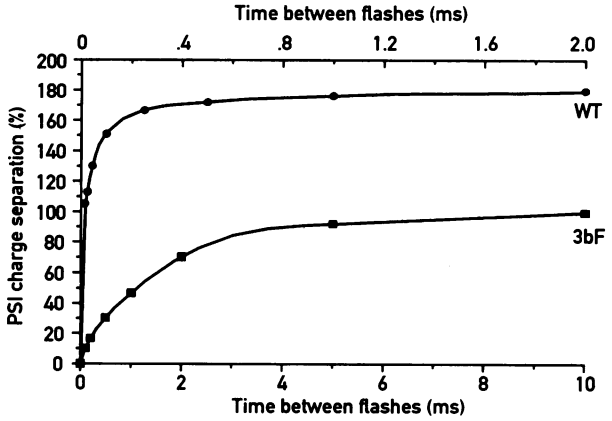

Fig. 6. Number of PSI charge separations induced by an actinic Xenon flash following a saturating laser flash given at time zero in WT (●) and 3bF (■). The data have been normalized to the signal induced by the laser flash taken as 100% of PSI charge separation. Note the different time scales for the mutant (lower, 0–10 ms) and for the WT (upper, 0–2 ms).

of a positive charge from P700 to cytochrome f, which induces the oxidation of a plastoquinol molecule at the Q_o site of the cytochrome b_6f complex and, subsequently, a transmembrane movement of electrons which increases the membrane potential (Joliot and Delosme 1974; Bouges-Bocquet, 1977). The kinetics of increase of this membrane potential following a saturating laser flash were measured for wild-type and 3bF (Figure 7) in the presence of the uncoupler FCCP, which was added in order to abolish the dark transmembrane electrochemical proton gradient. Figure 7 shows that, in wild-type, the increase in membrane potential is preceded by a lag phase of ~200 μs, which could be due to a limiting step between the oxidation of complexed plastocyanin and the subsequent oxidation of cytochrome f (Delosme, 1991). This lag is increased to

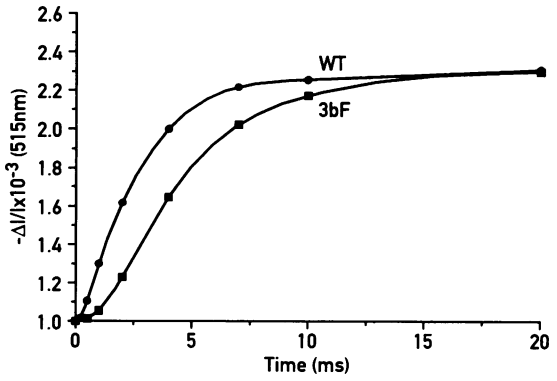

Fig. 7. Time course of the 515 nm absorption change measuring cytochrome oxidation induced by a saturating laser flash given at time zero. The curves for WT (●) and 3bF (■) are shown.

1.2 ms in 3bF and equals the longer turnover time of PSI centers observed in this mutant (Figure 7). In addition, the rate of the slow electrogenic phase is lower for the mutant than for the wild-type strain (slope of the curves in Figure 7). Thus, the transfer of a positive charge from P700 to cytochrome f is essentially limited by the rate of P700 reduction.

Discussion

Isolation of a psaF-deficient mutant of C.reinhardtii

One of the aims of this work was to explore the possibility of targeting inactivations to specific nuclear photosynthetic genes of *C.reinhardtii* through transformation and homologous recombination.

Nuclear gene targeting has been achieved in *C.reinhardtii* by transforming *nit1* mutant cells with a non-functional deletion derivative of the wild-type *nit1* gene and by selecting for growth on nitrate (Sodeinde and Kindle, 1993). The frequency of homologous to random integration events was estimated at 1:1000 with the glass-bead-mediated transformation. Smart and Selman (1991) first showed that transformation of *C.reinhardtii* can be used to generate mutations in specific nuclear genes. Although they succeeded in disrupting the nuclear *atpC* gene of *C.reinhardtii* by transforming cells with a non-functional *atpC* cDNA and an excess of foreign DNA and by using appropriate selection and screening procedures, the disruption did not occur through homologous recombination. This was not surprising as the transforming cDNA used was small and differed from the genomic sequence which most likely contains introns.

Here we have used a plasmid with a 3.8 kb genomic DNA fragment containing a derivative of the *psaF* gene that was inactivated by three small deletions. Transformation with this plasmid allowed us to recover a mutant with a large deletion covering the entire *psaF* gene. It is therefore possible that the mutation was created through homologous recombination followed by secondary DNA rearrangements that ultimately led to the formation of the deletion. Introduction of homologous DNA sequences into mammalian cells has been shown to induce mutations in the cognate gene (Thomas and Capecchi, 1986). We cannot exclude the possibility that the deletion in 3bF occurred by chance. However, whatever the exact origin of this

mutation is, this work shows that it is possible to isolate *C.reinhardtii* cells with defects in nuclear genes of specific PSI subunits, and most likely of subunits of other photosynthetic complexes, through nuclear co-transformation and by screening the transformants for altered fluorescence transients. It is noteworthy that this method has been successful even in the case of a subtle fluorescence phenotype, as shown here for the *psaF* mutant.

Phenotype of the psaF-deficient mutant of C.reinhardtii

A second aim of this work was to re-examine the phenotype of a *psaF* deficiency in a eukaryotic photosynthetic organism in light of the surprising result in cyanobacteria that disruption of *psaF* still allows for photoautotrophic growth with no apparent deficiency in photosynthetic activity (Chitnis *et al.*, 1991). In particular, no significant difference could be observed in the rate of reduction of $P700^+$ by cytochrome c553 with PSI particles isolated from either wild-type *Synechocystis* or the *psaF*-deficient mutant (Xu *et al.*, 1994). Yet biochemical work using eukaryotic organisms has clearly shown that the PsaF subunit interacts tightly and, most probably, specifically with soluble electron donors to PSI, such as the copper-containing protein plastocyanin and cytochrome c553 (Hippler *et al.*, 1989; Wynn and Malkin, 1988; Wynn *et al.*, 1989a,b).

The mutant strain 3bF of *C.reinhardtii* lacking the *psaF* gene is able to grow photoautotrophically, as observed for a *psaF*-deficient mutant of cyanobacteria (Chitnis *et al.*, 1991), indicating that in *C.reinhardtii*, the PsaF subunit is also dispensable for photosynthesis.

The interactions between plastocyanin and PSI are altered in this mutant. We observed a 20-fold decrease in the rate constant for the binding of plastocyanin to the PSI reaction center in the mutant relative to wild-type. Fast electron transfer between plastocyanin and P700 no longer occurs, probably because a plastocyanin–PSI complex competent for rapid electron transfer to $P700^+$ does not form appreciably under physiological conditions. Our results suggest that the PsaF polypeptide is directly involved in the docking of plastocyanin to the PSI reaction center as proposed earlier (Wynn and Malkin, 1988; Hippler *et al.*, 1989).

In the cyanobacteria *Synechocystis* PCC 6803 and *S.elongatus*, the reduction of photo-oxidized P700 follows essentially monophasic kinetics and lacks the fast kinetic component observed with higher plants and eukaryotic alga (Hatanaka *et al.*, 1993; Hervas *et al.*, 1995). This behavior is consistent with a bimolecular reaction between plastocyanin and PSI in which no stable complex is formed prior to electron transfer. The absence of fast electron transfer between plastocyanin and P700 in these cyanobacteria can be correlated with the absence of a region of their PsaF polypeptide that is conserved near the amino-terminal end in higher plants and eukaryotic algae, and that contains several positively charged residues (Hippler, 1994). This raises the possibility that this region mediates the interaction with negatively charged plastocyanin that leads to the formation of a stable plastocyanin–PSI complex responsible for fast electron transfer. The availability of the 3bF mutant allows one to test this hypothesis by site-directed mutagenesis.

It is noteworthy that the fluorescence transients of 3bF and wild-type are very similar in a young culture. This is compatible with the observation that the transfer of a positive charge from P700 to plastocyanin and cytochrome f is not diminished in the 3bF mutant in early exponential growth phase, but only slowed down (Figure 7). The rate for this process, 600 s^{-1}, remains 5–10 times faster than the rate constant of the reactions that limit the overall rate of the photosynthetic reactions, which is ~100 s^{-1} (Emerson and Arnold, 1932a,b). This explains why the 3bF mutant grows at a wild-type rate under photoautotrophic conditions. However, in older cultures, the fluorescence transients of 3bF change to a pattern indicating a failure to re-oxidize the plastoquinone pool. This property allowed us to isolate the 3bF mutant and indicates that the loss of the PsaF subunit has a secondary effect on PSI function in these cultures which has not been investigated further. Perhaps another essential function of PsaF which has also been conserved in cyanobacteria is to maintain the integrity and optimal activity of PSI under more adverse growth conditions.

Materials and methods

Strains and media

Escherichia coli: the *recA*$^-$ strains HB101 and DH5a were grown as described (Sambrook *et al.*, 1989). The Bl21(DE3) strain was used for overexpression of the recombinant PsaE and PsaF polypeptides.

Chlamydomonas reinhardtii: the cell wall-less arginine auxotroph cw15-arg7A was used for the nuclear transformation. This strain is also *nit1*$^-$, *nit2*$^-$. The wild-type strain used was *cw15*$^+$, *arg*$^+$, *nit*$^-$. Two PSI$^-$ mutant strains were used: H13 (Choquet *et al.*, 1992) and Δ4, isolated during the screening for *psaF* mutants. These strains were grown photoheterotrophically in TAP medium or photoautotrophically in HSM medium (Rochaix *et al.*, 1988). *nit*$^+$ strains were selected on Sager–Granick (SG) medium (Harris, 1989). When necessary, arginine was added at the final concentration of 90 μg/ml. All cultures of *C.reinhardtii* were grown at 25°C with appropriate illumination.

Isolation of a genomic fragment containing the psaF gene

The genomic *C.reinhardtii* cosmid library P was screened with a probe derived from the *psaF* cDNA, and the cosmid clone p21cos16 was isolated (Franzen *et al.*, 1989; Purton and Rochaix, 1994). The restriction map and the location of the *psaF* gene on the p21cos16 clone are shown in Figure 1A. The 3.8 kbp *Sph*I restriction fragment, 3.8F, containing the *psaF* gene was isolated and cloned in the pBS(+) vector (Stratagene) at the unique *Sph*I site of the polylinker, giving rise to the p3.8F1 plasmid (Figure 1B). Two subfragments of the *psaF* cDNA were used as probes: 21 Int and 21LGF (Figure 1B). The Gap-M probe is a genomic subfragment.

Construction of a mutated version of the psaF gene

A mutated version of *psaF* with small deletions in the 5′ and 3′ part of *psaF* and a gap in the middle of the coding sequence was constructed in order to inactivate the resident *psaF* gene of *C.reinhardtii* by nuclear transformation and gene targeting. This mutated version of *psaF* was constructed in three steps.

Step I. The 1.8 kbp *Bst*XI fragment of p3.8F1 encompassing the 5′ part of the *psaF* gene was cloned in the *Eco*RV site of pKS(–) after filling the ends with the Klenow enzyme. This plasmid was digested with *Xho*I, blunted with the Klenow enzyme, digested with *Eco*RI and the insert was cloned in the *Hin*dIII (blunted)–*Eco*RI sites of pBS(+) to give pB1.8XRF. This plasmid was digested with *Pst*I, blunted with the Klenow enzyme and then self-ligated to give pB1.8XRFΔP. This plasmid was cut with *Sac*II–*Nar*I, blunted with the Klenow enzyme and self-ligated to generate the plasmid pBFΔNS. This construct contains the 5′ part of the *psaF* gene, with a 127 bp deletion.

Step II. The 3.8F fragment was cloned in the pBS(+) vector in which the *Eco*RI–*Pst*I fragment of the polylinker was deleted. The *Nhe*I–*Bgl*II fragment from this plasmid was removed and replaced by the *Nhe*I–

*Bgl*II fragment derived from pB1.8XRFΔP. This new plasmid is called pΔBΔP3.8F

Step III. The *Bgl*II–*Msc*I fragment of pΔBΔP3.8F was deleted and replaced by the *Bgl*II–*Msc*I fragment of pBFΔNS (step I) giving rise to the pBΔFM5′ plasmid. This plasmid was finally digested with *Pst*I and the two larger fragments were isolated and religated together in the same original orientation. This plasmid, called p3.8FΔ4, has 100 bp deletions in the 5′ and 3′ regions of *psaF*, and a 200 bp gap in the middle of the *psaF* coding region. This plasmid was linearized at the *Msc*I restriction site and dephosphorylated with the CIP enzyme prior to nuclear transformations of algal cells.

Nuclear transformation of C.reinhardtii cells

C.reinhardtii cells were transformed by the glass bead method, as described by Kindle (1990), with slight modifications. Cells of the cw15-arg7A strain were grown nearly to saturation in TAP medium supplemented with arginine at a light intensity of 60 mE/m^2/s and then diluted into fresh medium. The cells were allowed to grow to a concentration of 2–3×10^6 cells/ml and then centrifuged at room temperature and resuspended in TAP medium at a concentration of 10^8 cells/ ml. About 3×10^7 cells were thoroughly vortexed for 15 s, in the presence of 300 mg of glass beads (0.45–0.5 mm diameter) and a total of 5 μg of DNA: 2.5 μg of the closed circular plasmid pArg7.8 harboring the ASL gene (Debuchy *et al.*, 1989) and 2.5 μg of the linearized dephosphorylated p3.8FΔ4 plasmid. Three ml of Top-TAP (0.6% agar in TAP medium) were added to the vortexed cells and poured on a TAP plate. The plates were incubated at 25°C under a light intensity of 0.75 mE/m^2/s. As a negative control, cells were vortexed in the presence of glass beads but in the absence of DNA.

Screening of the transformed algal cells for psaF mutants

The fluorescence transients of *arg*$^+$ cells were analyzed 15–17 days after the transformation. Plates were dark-adapted for 15 min. Fluorescence transients were determined with a video imaging system as described by Fenton and Crofts (1990). Photosynthetic mutant colonies were transferred to a fresh plate and incubated in dim light (0.75 μE/m^2/s). Total genomic DNA was then isolated from the photosynthetic mutants and tested by Southern analysis for alterations of the *psaF* locus by hybridization with the Gap-M probe, (Figure 1B). The only *psaF* algal clone isolated in this screen was called 3bF.

Rescue of the 3bF strain

The transformation method used for rescuing 3bF was the same as described above, except for the following: arginine was not included in the growth medium since the strain is prototrophic for arginine. The cells were transformed with 5 μg of the p3.8F1 plasmid harboring the active *psaF* gene (Figure 1B) as well as the pMN24 and pMN68 plasmids harboring the *nit1*$^+$ and *nit2*$^+$ genes respectively (Fernandez *et al.*, 1989; Schnell and Lefebvre, 1993). All the plasmids were in the supercoiled form. After vortexing the cells in the presence of glass beads and DNA, 3 ml of Top-SG (0.6% agar in SG) were added to the cells, and poured on SG plates. The plates were incubated at 25°C in high light (80 μE/m^2/s). The fluorescence transients of the transformants were analyzed and candidates were examined further by Southern and immunoblot analysis.

C.reinhardtii nucleic acid isolation and analysis

Total genomic DNA (minipreparations) was isolated from the algal cells as described (Rochaix *et al.*, 1988). Southern blottings were performed on GeneScreen (Dupont, NEN) membranes as described by the manufacturer. DNA probes were radioactively labeled by the random primed labeling method in the presence of [α-^{32}P]dATP (Feinberg and Vogelstein, 1984).

Total RNA was isolated from 50 ml cell cultures grown to a concentration of 3×10^6 cells/ml. The cellular pellet was resuspended in 4 ml of TEN (20 mM Tris–Cl pH 8; 50 mM EDTA; 0.1 M NaCl) and sonicated in the presence of one volume of phenol:chloroform: isoamylalcohol (25:24:1) solution for 30 s (maximal power for microtips). The RNA in the aqueous phase was precipitated with three volumes of ethanol. The clean dry pellet was resuspended in 0.1% SDS. RNAs were fractionated by agarose gel electrophoresis in phosphate buffer and blotted on GeneScreen membranes, as described above for DNA analysis.

Hybridization of membranes (either for Southern or Northern analysis) was performed as described (Rochaix *et al.*, 1988).

Overexpression of the PsaE and PsaF polypeptides in E.coli and antibody production

Recombinant PsaE and PsaF polypeptides were overexpressed in *E.coli*, using the pET vector expression system of Studier *et al.* (1990). The PsaE recombinant polypeptide had an N-terminal extension of six histidine residues (engineered in the pET 3c vector, kindly provided by L.Lopez-Molina). This histidine extension allowed us to purify the PsaE polypeptide by affinity chromatography on an agarose–NTA–Ni^{2+} column (Quiagen) as described (Stüber *et al.*, 1991). The full-length mature PsaF recombinant protein was found to sediment in the inclusion bodies of the overexpressing bacteria in a nearly homogeneous pure form. The recombinant PsaF polypeptide was solubilized in the presence of 6 M urea and further purified through a DEAE–Sepharose matrix (CL6B, Pharmacia). The purified recombinant polypeptides were injected into rabbits for antibody production.

Fluorescence transients, growth curves and Western analysis

Fluorescence transients were determined as described (Bennoun and Delepelaire, 1982). Cell concentration was determined with a hemacytometer. The cells were centrifuged and resuspended in TE buffer pH 8 (19 mM Tris–Cl pH 8; 1 mM EDTA), and the chlorophyll concentration was determined (Arnon, 1949). The samples were stored at –70°C. For Western analysis, aliquots were solubilized in sample buffer [62.5 mM Tris–Cl pH 6.8; 2.5% 2-mercaptoethanol; 2% SDS; 10% (v/v) glycerol], heated at 90°C for 1 min and fractionated by SDS–PAGE. Transfer to a nitrocellulose filter and incubation with antibodies were as described (Towbin *et al.*, 1979). The immune complexes were detected by enhanced chemiluminescence using the ECL kit (Amersham) or with [^{125}I]protein A (Harlow and Lane, 1988).

Spectroscopic measurements

Exponentially growing algal cells were centrifuged and resuspended in 20 mM Mes–NaOH pH 7/10% Ficoll at a concentration of 2.5×10^7 cells/ml and kept in anaerobic conditions in darkness which induce a full reduction of the plastoquinone pool and of the primary and secondary PSI donors. They were incubated with 3.5 μM FCCP in order to collapse the permanent membrane potential and to equilibrate the internal pH with that of the suspension buffer. Absorbance changes due to PSII redox changes were prevented by pre-illuminating the samples in the presence of 10^{-3} M hydroxylamine and 10^{-5} M DCMU (Bennoun, 1970). Total PSI charge separations were measured on a relative scale by determining field-indicating (electrochromic) absorbance changes at 515 nm induced by the electric field across the thylakoid membrane (Joliot and Delosme, 1974; Joliot and Joliot, 1985). The time interval between the actinic flash and the detecting flash was 100 μs. Under these conditions, the contribution of the short-lived carotenoid triplet state and of redox components such as P700 and cytochrome f to the absorbance changes can be neglected.

Oxidation of cytochrome f was measured under anaerobic conditions with a time interval of 30 s between the repetitive actinic flashes. Under these conditions, the two cytochromes b_l (low potential) and b_h (high potential) are oxidized and reduced, respectively, and the Q cycle predicts an electrogenicity around 1.3 (Joliot and Joliot, 1986).

Spectroscopic measurements were made at room temperature in a spectrophotometer similar to that described by Joliot and Joliot (1984), and modified as described (Joliot and Joliot, 1985).

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