The EMBO Journal vol.13 no.12 pp.2747-2754, 1994

The chloroplast *ycf8* open reading frame encodes a photosystem II polypeptide which maintains photosynthetic activity under adverse growth conditions

Caroline Monod[1], Yuichiro Takahashi[2], Michel Goldschmidt-Clermont and Jean-David Rochaix

Departments of Molecular Biology and Plant Biology, University of Geneva, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland and [2]Department of Biology, Faculty of Science, Okayama University, Okayama 700, Japan

[1]Present address: CNRS, Laboratoire de Microbiologie et Génétique Moléculaire, 118 Route de Narbonne, 31062 Toulouse cedex, France

Communicated by J.-D.Rochaix

We have engineered and analyzed a chloroplast mutant of *Chlamydomonas reinhardtii* that lacks *ycf8*, the chloroplast open reading frame 8, which is highly conserved in location and predicted amino acid sequence in land plants and *C.reinhardtii*. The *ycf8* sequence was replaced with the *aadA* cassette which confers resistance to spectinomycin when expressed in the chloroplast. Although the mutant is able to grow phototrophically, photosystem II function and cell growth are impaired under stress conditions such as high light intensity and diminished chloroplast protein synthesis induced by spectinomycin. Use of an antibody generated against the *ycf8* product has revealed that this hydrophobic polypeptide is associated with photosystem II, based on its severely reduced levels in various photosystem II-deficient mutants and on its copurification with photosystem II. This protein, therefore, appears to be (i) a novel photosystem II subunit and (ii) required for maintaining optimal photosystem II activity under adverse growth conditions.

Key words: chloroplast open reading frame/photosystem II/stress

Introduction

The four major photosynthetic complexes, photosystem II (PSII), photosystem I (PSI), the cytochrome b6-f complex and the ATP synthase are involved in the primary reactions of photosynthesis. They comprise numerous chloroplast- and nuclear-encoded subunits. Whereas the functional roles of most of the larger subunits of these complexes have been elucidated, the functions of the small molecular weight subunits remain largely unknown (for review see Erickson and Rochaix, 1991; Vermaas, 1993). N-terminal sequences of several small subunits of PSII and PSI have been found to correspond to small ORFs (open reading frames) of the chloroplast genome whose entire sequence has been determined in tobacco (Shinozaki *et al.*, 1986), liverwort (Ohyama *et al.*, 1986), rice (Hiratsuka *et al.*, 1989) and *Euglena gracilis* (Hallick *et al.*, 1993). These chloroplast DNA sequences have revealed the existence of many small ORFs which may encode functional polypeptides.

A small chloroplast ORF located downstream of, and co-transcribed with, *psbB* has been found in *Chlamydomonas reinhardtii* (Monod *et al.*, 1992). This ORF, designated *ycf8* for 'hypothetical chloroplast open reading frame 8', is evolutionarily conserved from *C.reinhardtii* throughout all higher plants examined and it could encode a polypeptide between 31 and 38 amino acids. While the organization of most protein-encoding chloroplast genes in *C.reinhardtii* differs from that found in higher plants, a striking exception is provided by the *psbB* region in which the relative arrangement of *psbB*, *ycf8*, *psbN* and *psbH* is conserved (Monod *et al.*, 1992; Johnson and Schmidt, 1993). However, in contrast to higher plants where *petB* and *petD* are part of the *psbB* operon, these genes are located elsewhere on the chloroplast genome of *C.reinhardtii* (Büschlen *et al.*, 1991).

All homologs of *ycf8* encode a negatively charged glutamic acid as the second residue, a hydrophobic central region and several positively charged amino acids in the C-terminal region (Monod *et al.*, 1992). This ORF, therefore, has the potential to encode a small membrane-spanning polypeptide. Furthermore, since *ycf8* is co-transcribed with one of the principal polypeptides of the PSII complex, it has been tempting to speculate that the *ycf8* product is associated with, and of functional importance to, this photosynthetic complex.

Here we demonstrate that *ycf8* is expressed as part of the *psbB-psbH* operon and that its product is specifically associated with the PSII complex and, thus, represents a new photosystem II subunit. We also show that inactivation of *ycf8* puts the PSII complex at a disadvantage under certain stress conditions, such as high light and reduced chloroplast protein synthesis induced by spectinomycin. This is the first identification of a small PSII subunit that appears to be essential for maintaining high photosynthetic activity under adverse growth conditions.

Results

Construction of a chloroplast ycf8 deletion mutant

To investigate the function of *ycf8* in *C.reinhardtii*, this ORF was deleted by replacement with the chimeric *aadA* gene, coding for aminoglycoside adenyl transferase, which confers resistance to the chloroplast protein synthesis inhibitor spectinomycin (Goldschmidt-Clermont, 1991). The *aadA* gene was inserted in both orientations downstream of *psbB*, and each of these constructs was used to transform a wild-type strain of *C.reinhardtii* with a particle gun. The constructs used are shown in Figure 1. To assess possible secondary effects due to the presence of spectinomycin, the *aadA* expression cassette was inserted upstream of *psbD* at a site which does not disrupt any essential photosynthetic function. The corresponding transformant, called A, was used as a control. The transformants were selected for growth on plates containing acetate and spectinomycin.

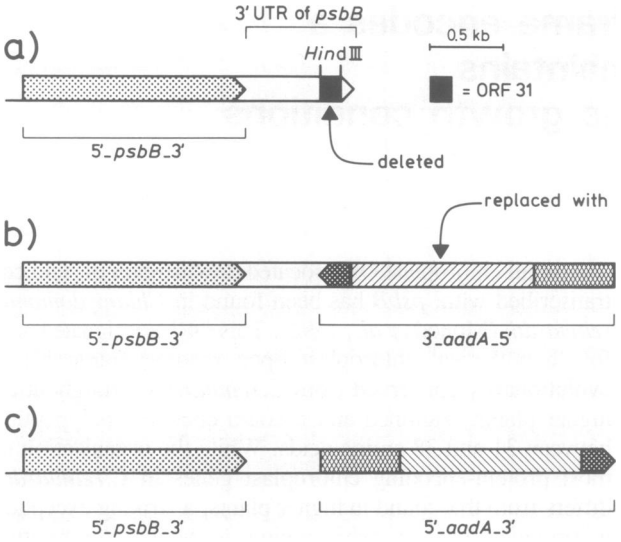

Fig. 1. Constructs used for transformation. Diagrammatic representation of (**a**) the endogenous *psbB* gene with the 3′ untranslated region of *psbB* containing *ycf8* (ORF31) and (**b** and **c**) the construct with *ycf8* deleted and replaced with the *aadA* cassette in the opposite and the same orientation relative to *psbB*. The *aadA* cassette consists of the *aadA* coding sequence fused at its 5′-end to the *atpA* promoter and leader region and at its 3′-end to the 3′ untranslated region of *rbcL* (Goldschmidt-Clermont, 1991). The black stippled on white regions represent the 5′ untranslated and coding regions of *psbB*. The white region represents the 3′ untranslated region of *psbB*. The white stippled on black region indicates the 3′-end of *rbcL*. The slashed rectangle represents the *aadA* coding sequence. The crosshatched rectangle indicates the 5′ leader and promoter region of *atpA*.

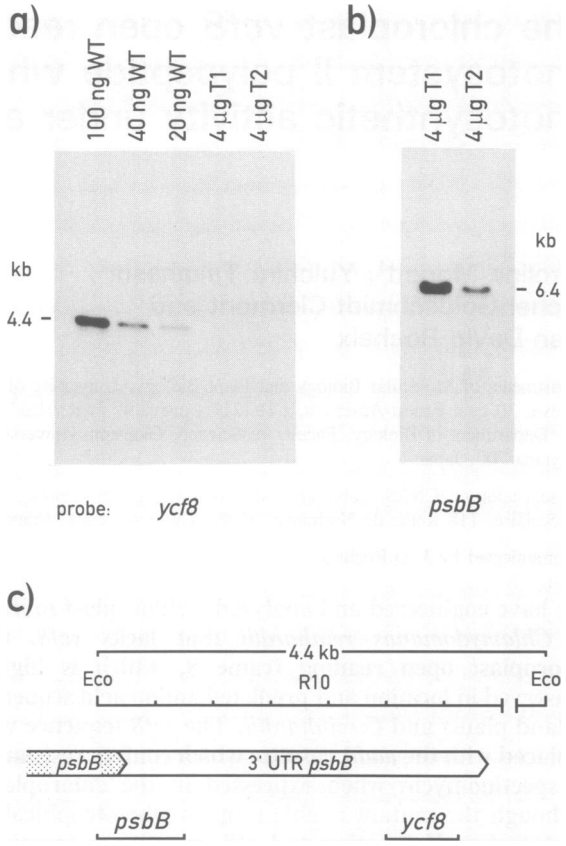

Fig. 2. Southern analysis of the transformants T1 and T2 lacking *ycf8*. The amounts and types of *Eco*RI-digested DNA loaded are indicated. WT, wild-type. The probe in (**a**) was the 140 bp *Hin*dIII fragment containing *ycf8* that was deleted from the transformants T1 and T2. The probe used in (**b**) was a 160 bp *Eco*RI–*Afl*III fragment comprising the last 50 coding bases of *psbB* and the first 110 bases of the 3′ untranslated region of *psbB*. The same filter was used sequentially for both probes. (**c**) A diagram showing the locations of the probes used.

Because each *C.reinhardtii* cell contains ~80 copies of the chloroplast genome, it was important to demonstrate that the transformants were homoplasmic, i.e. that every *ycf8* copy had been removed by replacement with the *aadA* cassette. After recloning the transformants three times to allow segregation of the chloroplast genomes, Southern analysis was performed. It can be seen in Figure 2a that hybridization of the *Eco*RI-digested DNA from two transformants, T1 and T2, with a probe specific for *ycf8* does not give rise to a signal under conditions where wild-type DNA, 200-fold diluted, produces a band at 4.4 kb corresponding to *Eco*RI fragment R10 (Figure 2c). Hybridization with a *psbB* probe (Figure 2b) reveals the presence of a 6.4 kb *Eco*RI fragment in the transformants, as expected from the insertion of the 1.9 kb *aadA* cassette within the R10 fragment (Figure 2c).

Replacing ycf8 with the aadA cassette affects transcription patterns downstream of psbB

Since the introduced *aadA* cassette contains the 3′-end of *rbcL* of *C.reinhardtii* that is known to have a processing site or transcriptional stop signal (Goldschmidt-Clermont, 1991), it was necessary to determine whether replacing *ycf8* with the *aadA* cassette had perturbed in any way the appearance of transcripts encoded downstream of *ycf8*. Northern analysis of the transformants with the *aadA* sequence in either orientation is shown in Figure 3. The probes were the 4.4 kb *Eco*RI fragment covering the *psbB* downstream region and the 1.1 kb *Eco*RI–*Dde*I fragment that corresponds to most of the *psbB* coding sequence (Figure 3c). Previous work demonstrated the existence of two *psbB* mRNAs of 2.3 and 1.7 kb (Monod *et al.*, 1992). The first includes *psbB* and *ycf8*, while the second has a shorter 3′ untranslated region and contains only *psbB*. In the transformants T1 and T2, in which the *aadA* cassette is oriented in the opposite direction to the upstream *psbB* gene, the transcripts detected with the 4.4 kb probe are the same as in wild-type, except for the loss of a 2.3 kb RNA (Figure 3a). The level of the shorter 1.7 kb *psbB* RNA is considerably enhanced (Figure 3b). The faint signals of ~2.3 kb in Figure 3b (lanes T1, T2, T3 and T4) could represent *psbB* transcripts that terminate within the *aadA* cassette. The 0.9 and 0.5 kb transcripts seen in the wild-type and the transformants T1 and T2 (Figure 3a) are derived from within the 1.9 kb *Sca*I–*Eco*RI fragment downstream of *psbB* (Monod *et al.*, 1992; see Figure 3c); these transcripts have since been determined to correspond to *psbH* (Johnson and Schmidt, 1993). However, when *aadA* is oriented in the same direction as *psbB*, only the 1.7 kb *psbB* transcript accumulates and the levels of the 0.9 and 0.5 kb transcripts are strongly diminished (see Figure 3a and b, lanes T3 and T4). Under these conditions, the 3′ region of the *aadA* cassette presumably acts as a transcription terminator or processing site and prevents the normal accumulation of downstream transcripts (see Figure 3c). These data strongly suggest that transcription begins upstream of *psbB* and proceeds through *ycf8* towards the downstream region containing *psbH*.

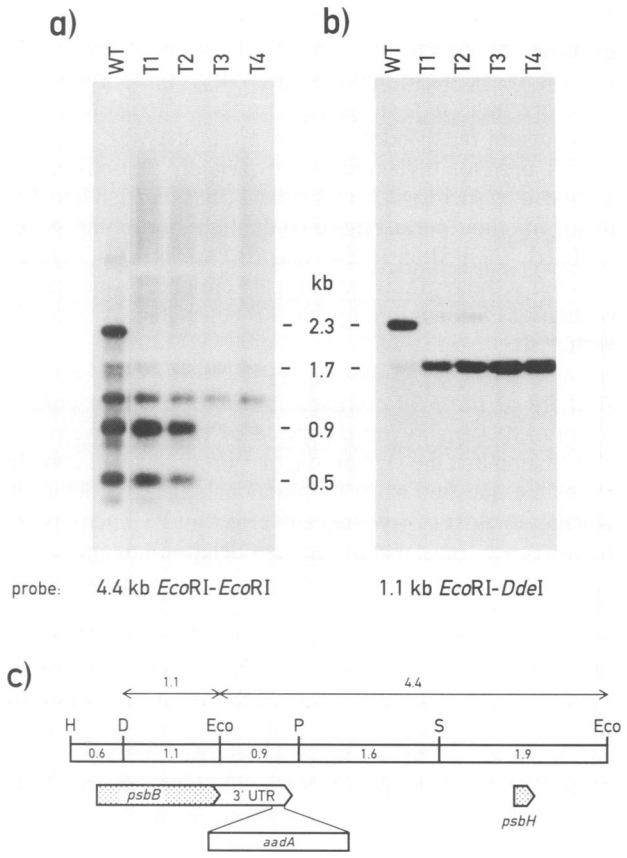

Fig. 3. Northern analysis of the transformants lacking *ycf8*. Total RNA was hybridized with the 4.4 kb *Eco*RI fragment as probe that includes the 3′ untranslated region of *psbB* and the downstream region with *psbH* (**a**) and with the 1.1 kb *Eco*RI fragment that contains exclusively *psbB* coding sequence (**b**). (**c**) Diagram of the *psbB* region. Note that the 1.7 kb transcripts detected in (a) and (b) are distinct (Monod *et al.*, 1992).

Because the aim of these experiments was to determine the function of *ycf8* itself, only the transformants T1 and T2 with the *aadA* sequence in the opposite orientation with respect to *psbB* were analyzed further so as to avoid the polar effects on downstream transcripts.

ycf8 is expressed and encodes a thylakoid polypeptide

In *C. reinhardtii*, *ycf8* encodes a potential protein of 31 amino acids with an extensive hydrophobic region (Monod *et al.*, 1992). To test whether such a polypeptide exists in the chloroplast, *ycf8* was expressed in *Escherichia coli* and the recombinant protein was used to generate antibodies. Whole cell proteins, thylakoid membrane polypeptides and soluble cell proteins from wild-type, strain A and the transformant T1 were fractionated by PAGE, blotted to nitrocellulose filters and probed with antibodies against D1, one of the PSII reaction center subunits and the *ycf8* polypeptide. Figure 4 shows that both proteins are present in the thylakoids of wild-type and the A strain. No signal is detectable with the supernatant fraction, indicating that the *ycf8* protein is associated with thylakoid membranes. As expected, it is absent from the thylakoids of the transformant T1 which nevertheless accumulates D1.

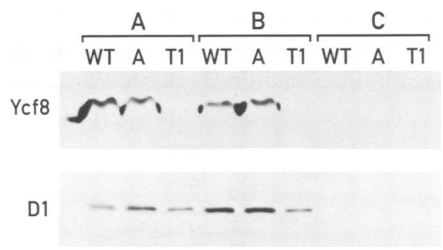

Fig. 4. The *ycf8* product is a thylakoid polypeptide. Western analysis of (A) whole cell proteins, (B) thylakoid membrane proteins and (C) soluble cell proteins from wild-type, strain A (control) and transformant T1. The blots were reacted with D1 and *ycf8* antibodies.

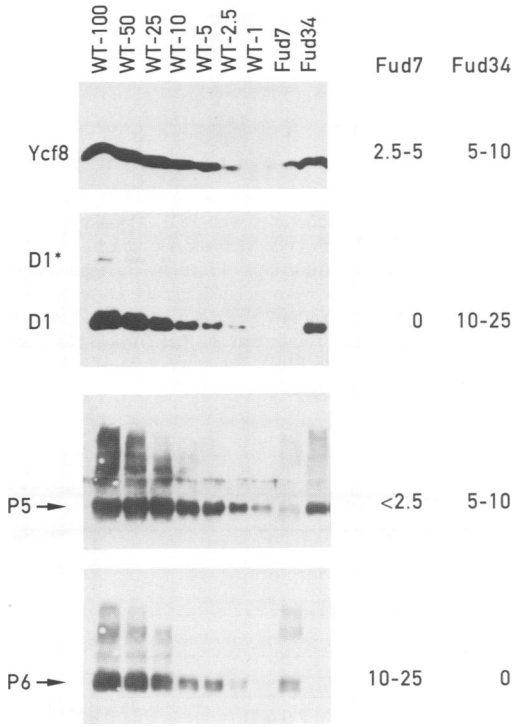

Fig. 5. The *ycf8* product is associated with PSII. The protein blots from wild-type and the PSII mutants Fud7 and F34 were reacted with antibodies against *ycf8* protein, D1, P5 and P6. Equal amounts of chlorophyll (5 μg) were loaded on each lane. Dilutions from wild-type were used to obtain a quantitative estimate of the amount of *ycf8* protein in the PSII mutants. The amount of each protein accumulated in Fud7 and Fud34 is indicated on the right in percentage of wild-type levels. D1* indicates the aggregated form of the D1 protein. Arrows indicate the bands corresponding to P5 and P6.

The ycf8 product is associated with photosystem II

The presence of the *ycf8* product within the thylakoid membrane raises the question whether it is associated with a particular photosynthetic complex. We therefore examined several mutants known to lack a single photosynthetic complex for the presence of the *ycf8* polypeptide by Western analysis.

The results of this analysis revealed that, while this polypeptide accumulates to the wild-type level in H13, deficient in PSI (Choquet *et al.*, 1988) and Fud6, lacking the cytochrome b6−f complex (Lemaire *et al.*, 1986) (data not shown), it is strongly reduced in Fud7 and Fud34 (Bennoun *et al.*, 1986; Rochaix *et al.*, 1989), two mutants deficient in PSII (Figure 5). To obtain a quantitative estimate of the amount of *ycf8* product accumulated in the PSII

mutants, wild-type extracts were diluted several-fold and the signals compared (Figure 5). The amount of *ycf8* product accumulated in Fud7 and Fud34 is between 2.5 and 5, and 5 and 10%, respectively. These levels are in the same range as those obtained for the other PSII core polypeptides D1, P5 and P6 in these mutants (de Vitry *et al.*, 1989), and therefore indicate that the *ycf8* protein belongs to PSII.

To obtain independent evidence that the *ycf8* protein is a PSII protein, thylakoids were partially solubilized and separated by sucrose density gradient centrifugation into three major fractions consisting of (i) the light harvesting system (LHCII), (ii) PSI and PSII, and (iii) solely PSI. Figure 6 shows that the *ycf8* product is highly enriched in the fraction containing PSII, confirming that this polypeptide is associated with PSII.

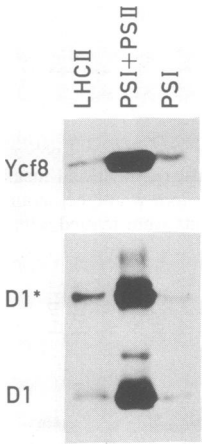

Fig. 6. The *ycf8* product fractionates with PSII. Thylakoid membranes were partially solubilized with dodecyl maltoside and fractionated by sucrose density gradient centrifugation. Fractions corresponding to LHCII, PSI + PSII and PSI were isolated and the corresponding proteins were analyzed by Western blotting using antibodies against *ycf8* product and D1 (which shows that the LHCII and PSI fractions are slightly contaminated with PSII). Equal amounts of chlorophyll (5 μg) were loaded on each lane.

Activity of the PSII complex is affected in the ycf8 deletion mutant in the presence of spectinomycin

The finding that the *ycf8* product is part of the PSII complex raises the question as to its functional role. To determine whether the loss of *ycf8* had any effect on PSII activity, the fluorescence transients of the *ycf8* deletion mutants and of the control strain were measured either in the presence or absence of spectinomycin. In all cases, measurements were also performed in the presence of the herbicide diuron to ensure that the fluorescence induction reached saturation. It can be seen in Figure 7 that in the absence of spectinomycin the fluorescence transients in the transformants T1 and T2 lacking *ycf8* are only mildly affected relative to the control which displays a typical wild-type pattern. In the

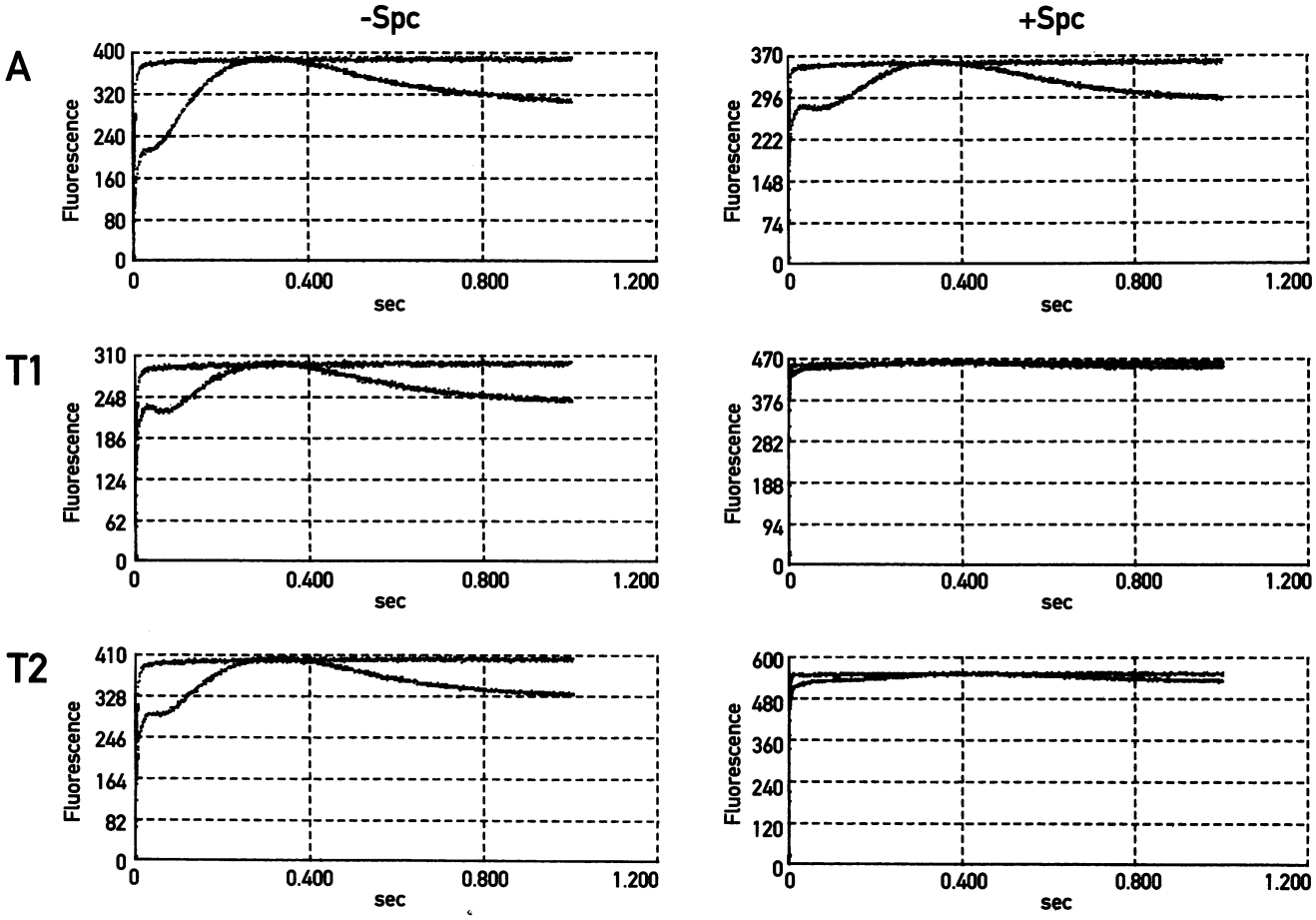

Fig. 7. Reduced variable fluorescence in the transformants T1 and T2 in the presence of spectinomycin (Spc). Fluorescence transients of the transformants A (control), T1 and T2 grown without and with spectinomycin (50 or 100 μg/ml) in liquid culture. In each panel the upper curve represents the measurement in the presence of 10^{-5} M diuron.

presence of spectinomycin, however, the fluorescence transients of the transformants display considerably less variable fluorescence than in the control.

A more quantitative measure of photosynthetic activity is provided by the ratio between variable fluorescence and maximum fluorescence F_v/F_{max}. Table I shows that while this ratio is slightly diminished in the absence of spectinomycin in the transformants T1 and T2, relative to the control A, in the presence of spectinomycin this ratio is considerably lower in the transformants than in the control. Measurements of O_2 evolution activity revealed a slight decline in the transformant T1 relative to the control A in the absence of spectinomycin (Table I). This difference between T1 and A was not significantly enhanced under weaker illumination (0.2 $\mu E.m^{-2}s^{-1}$; data not shown). A 2-fold decrease of O_2 evolution activity occurred in control cells grown with spectinomycin (Table I). In agreement with the fluorescence transients, O_2 evolution was significantly lower in the mutant T1 than in the control strain when the cells were grown with spectinomycin, indicating a synergistic effect of spectinomycin and *ycf8* deficiency on PSII activity. The growth rate of the transformants under phototrophic growth conditions with spectinomycin was reduced 2-fold relative to the control, whereas in the absence of the drug the transformants grew at the same rate as the control (data not shown).

The fluorescence transients and oxygen evolution activity measurements indicate that *ycf8* deficiency affects PSII function mostly in cells grown with spectinomycin. To determine whether these effects are due to alterations in the levels of PSII subunits, including *ycf8* protein, Western blot analysis was performed on the control strain A and the *ycf8*-deficient transformants T1 and T2 in the absence or presence of spectinomycin. Although these strains are spectinomycin-resistant, chloroplast protein synthesis is probably partially inhibited by the drug. The levels of the PSII reaction center subunit D1 and the PSII core subunits P5 and P6 are reduced at least 4-fold in A, T1 and T2 grown with spectinomycin relative to the untreated cells (Figure 8). In contrast, the amount of *ycf8* polypeptide in A is the same or even slightly increased in the presence of the drug (Figure 8). The levels of other chloroplast proteins, such as the PSI subunit PsaF and the ribosomal protein L1, are also unaffected by spectinomycin. This specific decrease of the PSII core polypeptides may be due to the fact that D1 turnover and PSII repair become limiting when chloroplast protein synthesis is reduced by spectinomycin. Thus, it appears that under these conditions the accumulation of the *ycf8* product is not affected by the reduced levels of the PSII core polypeptides, whereas in the PSII-deficient mutants examined the levels of these polypeptides and of the *ycf8* product are diminished to the same extent (Figure 5).

An important point shown in Figure 8 is that the levels of the PSII core polypeptides D1, P5 and P6 are the same in A, T1 and T2 in the presence of spectinomycin. Thus, *ycf8* deficiency cannot be exacerbating the PSII-defective phenotype through an effect on the accumulation of the PSII subunits or PSII stability.

Growth of the ycf8 deletion transformant is impaired under high light

The observation that the *ycf8*-deficient transformant grows photoautotrophically and that its photosynthetic function is only affected under adverse growth conditions in the presence of spectinomycin suggested that the function of the *ycf8* product may also become apparent under other stress conditions. To test this possibility, cells were grown on both acetate and minimal media without spectinomycin and under high or low light (1200 and 90 $\mu E/m^2/s$, respectively). It can be seen in Figure 9 that growth of the *ycf8* deletion transformant is severely reduced under high light both in the presence (TAP) and absence (MIN) of acetate, but not under low light.

Discussion

The ycf8 protein is associated with photosystem II

We have characterized a chloroplast ORF, *ycf8*, and shown that this ORF is expressed giving rise to a small thylakoid polypeptide. The latter has been identified as a small molecular weight PSII subunit, based on its reduced level in several mutants unable to stably accumulate the PSII complex, and on its co-fractionation with PSII. *ycf8* should therefore be designated as *psbS* in accordance with the international gene nomenclature. In recent years the number of identified PSII subunits has increased steadily, especially amongst the small molecular weight subunits. The PSII complex is presently considered to consist of at least 13 chloroplast- and four nuclear-encoded subunits (Erickson and Rochaix, 1991; Vermaas, 1993). With the exception of the larger *psbA*, *psbD*, *psbB* and *psbC* subunits which are part of the PSII core complex, the role of most of the smaller subunits remains poorly understood.

Analysis of several PSII mutants from *C.reinhardtii* has revealed that loss of any of the larger PSII core subunits leads to increased turnover of the other PSII subunits (Bennoun *et al.*, 1986; Jensen *et al.*, 1986; Kuchka *et al.*, 1988; de Vitry *et al.*, 1989). A similar PSII destabilization has also been observed recently in transformants of *C.reinhardtii*, with disruption of the *psbK* and *psbI* genes

Table I. Photosynthetic activity of the *ycf8::aadA* mutants

	F_v/F_{max}		O_2 evolution (μmol O_2/mg chl/h)	
	−Spc	+Spc	−Spc	+Spc
A	0.75 ± 0.01	0.48 ± 0.01	66 ± 4	34 ± 2
T1	0.54 ± 0.03	0.20 ± 0.04	59 ± 3	24 ± 2
T2	0.58 ± 0.04	0.21 ± 0.04	nd	nd

F_v, variable fluorescence; F_{max}, maximum fluorescence; Spc, spectinomycin; nd, not determined. Four independent measurements of fluorescence transients and O_2 evolution were performed. O_2 evolution measurements were carried out under an illumination of 30 $\mu E/m^2/s$.

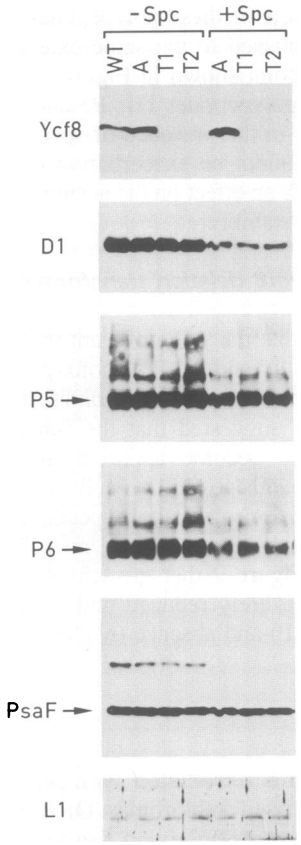

Fig. 8. Growth in the presence of spectinomycin leads to a decrease in the levels of the PSII core polypeptides, but not in the levels of the *ycf8* protein. Western analysis of thylakoid membranes from wild-type and transformants A, T1 and T2 grown without or with spectinomycin (100 μg/ml). The protein blots were reacted with antibodies against *ycf8* protein, D1, P5, P6, *psaF* protein and L1 protein. Arrows indicate the bands corresponding to P5, P6 and the psaF product.

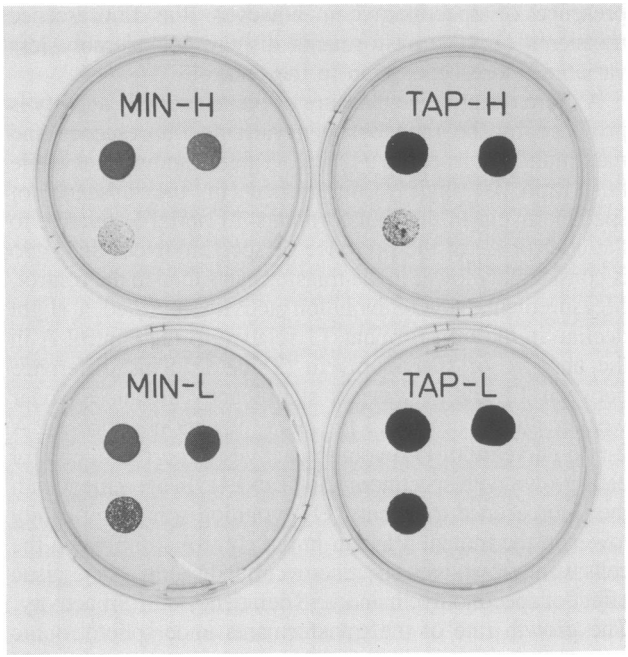

Fig. 9. Growth of the *ycf8* deletion mutant is impaired under high light. Aliquots of cells from wild-type (upper left), transformant A (upper right) and T1 (lower left) were spotted on TAP and MIN agar plates and grown for 36 h either under high (H, 1200 $\mu E/m^2/s$) or low light (L, 90 $\mu E/m^2/s$).

(Takahashi *et al.*, 1994; P.Künstner, A.Guardiola, Y.Takahashi and J.-D.Rochaix, unpublished results). However, similar gene disruptions do not affect the stability and the photochemical activity of PSII in cyanobacteria (Ikeuchi *et al.*, 1991). Here we have shown that the *ycf8* deletion mutants accumulate the D1 protein to wild-type levels, indicating that the *ycf8* product is not required for the stability of the complex. Another PSII subunit whose absence does not lead to destabilization of PSII in *C.reinhardtii* is the nuclear-encoded *psbP* product (Mayfield *et al.*, 1987). However, in this case the O_2-evolving activity of PSII is reduced to 5%, while in the *ycf8* deletion transformant this activity is unaffected under normal growth conditions.

The amount of *ycf8* product is reduced to the same extent as the other PSII core polypeptides in PSII mutants deficient in PSII complex accumulation (Figure 5). However, under conditions of diminished chloroplast protein synthesis induced by spectinomycin in cells carrying the *aadA* expression cassette, the *ycf8* product is maintained at wild-type levels while the amount of the PSII core polypeptides is reduced at least 4-fold (Figure 8). This apparent conflict may be due to the fact that in the PSII mutants a proper PSII complex can never assemble because of the lack of one of the core subunits. In these mutants the *ycf8* product may not insert stably into the thylakoid membrane. In the presence of spectinomycin intact PSII is formed, although at a reduced level presumably because of D1 turnover. This suggests that the stable accumulation of the *ycf8* product depends on an intact PSII core and that its level is not affected by the turnover of the PSII core subunits.

As in other land plants, *ycf8* is co-transcribed with *psbB* in *C.reinhardtii*. With the exception of the ribosomal operon, the *psbB* operon is the only one that has been conserved, at least partially, in *C.reinhardtii* and other plants. This conservation points to the importance of co-expression of the *psbB* and *ycf8* products. It is noteworthy that *psbH*, which has been mapped downstream of *psbB* (Johnson and Schmidt, 1993), also appears to be co-transcribed with *psbB* and *ycf8* based on the results obtained with the *ycf8* replacements with the *aadA* expression cassette (Figure 3). It appears, therefore, that the transcriptional organization of the *psbB* region is remarkably similar between *C.reinhardtii* and plants.

The functional role of ycf8 is apparent only under adverse growth conditions

The sequence of the *ycf8* product has been highly conserved between *C.reinhardtii* and other photosynthetic organisms. It contains a central hydrophobic region which may anchor this polypeptide within the thylakoid membrane. Transformants lacking *ycf8* are still able to grow photoautotrophically at the same rate as wild-type. However, their photosynthetic function is slightly impaired, as revealed by the reduction of the F_v/F_{max} ratios, although the yield of oxygen evolution is unaffected.

It is only under certain adverse growth conditions that a difference between transformant and wild-type becomes apparent. In the presence of spectinomycin, which presumably leads to a decrease of chloroplast protein synthesis even in cells carrying the *aadA* expression cassette,

photosynthetic function is affected at the level of PSII as measured by fluorescence transients and oxygen evolution. A possible function for the *ycf8* protein may be to maintain the photochemical efficiency of PSII under conditions of reduced chloroplast protein synthesis.

Growth under high light provides another condition where a difference between wild-type and the *ycf8* deletion mutant is apparent, independent of the presence of spectinomycin. A clear impairment of growth of the mutant occurs in high light. One possibility is that the *ycf8* protein is required for efficient replacement of the D1 protein which is known to turn over rapidly under strong illumination (Barber and Andersson, 1992). Since the *ycf8* protein appears to span the thylakoid membrane, it could help maintain thylakoid membrane integrity and structure around the PSII complex. In conclusion, the *ycf8* product has properties of a photosynthetic stress protein because its role manifests itself only under certain stress conditions, such as reduced chloroplast protein synthesis and high light. However, in contrast to usual stress proteins that are expressed only under stress conditions, the *ycf8* protein appears to be expressed constitutively.

Materials and methods

DNA constructs

Procedures for preparation of recombinant DNA plasmids are described by Sambrook *et al.* (1989). A 4.1 kb *Hin*cII fragment, that includes the entire *psbB* gene and 3′ UTR, was subcloned into the plasmid vector pBluescript SK$^-$ (Stratagene) and digested with *Sma*I and *Hin*cII to remove the resident *Hin*dIII site and to provide two blunt ends for the 4.1 kb *Hin*cII fragment. The resulting recombinant plasmid was digested with *Hin*dIII which cleaved twice to yield a 140 bp fragment containing ORF 31 (*ycf8*) and the remaining plasmid. The latter was treated with Klenow enzyme and used to insert the *aadA* expression cassette conferring spectinomycin resistance, which was excised from the plasmid pUC-atpX-AAD (Goldschmidt-Clermont, 1991) by digesting with *Sma*I and *Eco*RV. Restriction enzyme analysis confirmed that the *aadA* sequence was introduced in either orientation in various subclones.

Transformation

Recombinant plasmids with the *aadA* sequence in either orientation were used to transform a wild-type *Chlamydomonas* strain with a particle gun, as described by Zumbrunn *et al.* (1989) and Goldschmidt-Clermont *et al.* (1991).

Southern analysis

The DNA used for Southern analysis of the transformants was prepared from cells grown in Tris−acetate phosphate (TAP) medium (Harris, 1989) and light (~150 $\mu E/m^2/s$) according to the procedure described by Weeks *et al.* (1986). DNA from transformants was digested with *Eco*RI according to Sambrook *et al.* (1989). Southern analysis was performed with Amersham Hybond N+ membrane according to the manufacturer's protocol, with the modification of adding 1% SDS in the prehybridization, the hybridization and all the washes. The final wash was 0.5× SSPE and 1% SDS at 65°C.

Northern analysis

Total RNA was prepared according to a minipreparation procedure described in Goldschmidt-Clermont *et al.* (1990). Cells were grown in TAP medium in bright light (~150 $\mu E/m^2/s$), TAP medium in dim light (~0.3 $\mu E/m^2/s$) or high salt minimal (MIN) medium in bright light, in the absence or presence of spectinomycin at 20, 40 or 100 $\mu g/ml$.

Fluorescence transients and oxygen evolution

25 ml of liquid media, with or without spectinomycin, were inoculated with cells from a TAP plate containing spectinomycin and grown in dim light (5 $\mu E/m^2/s$) for 3 days. Cultures with a normal appearance and not exceeding 3.5×10^6 cells/ml were then diluted to 0.5×10^6 cells/ml. The *ycf8* deletion mutants grew more slowly than the control strain in the presence of spectinomycin and therefore were not, or were less, diluted than the control strain. Approximately 24 h after this dilution, when all cultures averaged between 2 and 3×10^6 cells/ml, fluorescence transients were recorded using the shutterless system [time resolution, 10 μs; Plant Efficiency Analyzer (PEA) by Hansatech Instruments, UK] described by Strasser and Govindjee (1991). To measure the fluorescence transients of cells from liquid cultures, aliquots were removed from the shaking cultures and immediately placed in small glass vials and incubated in the dark for ~5 min. In some cases the herbicide DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], also called diuron, was added to 10 μM. For liquid cultures, cells were grown with shaking in TAP medium and light (150 $\mu E/m^2/s$) without or with spectinomycin (100 $\mu g/ml$). Cells were also grown on agar-solidified TAP medium and bright light without or with spectinomycin (150 $\mu g/ml$). Cells growing on agar-solidified plates were incubated for 10 min in the dark prior to measuring fluorescence transients according to Fenton and Crofts (1990). The results were consistent between liquid TAP and solid TAP media when utilizing spectinomycin concentrations of 100 and 150 $\mu g/ml$, respectively.

Oxygen-evolving activity was measured as described (Takahashi *et al.*, 1994). Cells were used at a concentration of $1-3\times10^6$ cells/ml and measurements were performed at a light intensity of 30 and 0.2 $\mu E/m^2/s$.

Antibody production

ycf8 was inserted into the pET-3a vector described by Studier *et al.* (1990) and expressed in *E.coli* according to Sambrook *et al.* (1989). The recombinant 4.7 kDa protein was purified using the discontinuous gel system described by Schägger and von Jagow (1987), eluted and injected into rabbits. Antibodies against P5 and P6 were generously provided by N.H.Chua, those against D1 by L.McIntosh, those against the ribosomal protein L1 by J.Boynton and N.Gillham and those against PsaF by J.Farah.

Western analysis

Whole cell proteins, thylakoid proteins or soluble cell proteins were used. Thylakoids were prepared as described (Chua and Bennoun, 1975), with the addition of protease inhibitors after disruption of cells with a French pressure cell. Cells from the *ycf8* deletion mutants T1 and T2 and the spectinomycin-resistant control strain A were grown in TAP medium under 150 $\mu E/m^2/s$ of light with or without spectinomycin (100 $\mu g/ml$). Separation of three fractions (LHCII, PSI and PSII, and PSI) was performed by sucrose density gradient centrifugation as described (Takahashi *et al.*, 1991). Proteins were separated by SDS−PAGE according to Schägger and von Jagow (1987). Proteins were blotted onto nitrocellulose filters (BA83, Schleicher and Schuell) or onto Hybond-C extra membrane (Amersham). The blotting solution contained 25 mM Tris, 240 mM glycine, 0.05% SDS and 20% methanol. Signals were detected by enhanced chemiluminescence (ECL) according to the protocol of Amersham, except that 5% non-fat milk was added to the primary antibody solution. Alternatively, the alkaline phosphatase method was used (Boehringer Mannheim Biochemica).

Acknowledgements

We wish to thank W.Zerges for helpful comments, N.Roggli for skilled preparation of the figures, J.Bouverat for expert handling of the rabbits, Drs N.H.Chua, L.McIntosh, J.Boynton and N.W.Gillham and J.Farah for antibodies. We also thank Dr R.J.Strasser for his generous help and the loan of the PEA system for measuring chlorophyll fluorescence transients. This work was supported by grant 31.34014.92 from the Swiss National Fund and by a grant from the Human Frontier Science Program.

References

Barber,J. and Andersson,B. (1992) *Trends Biochem. Sci.*, **17**, 61−66.
Bennoun,P., Spierer-Herz,M., Erickson,J., Girard-Bascou,J., Pierre,Y., Delosme,M. and Rochaix,J.-D. (1986) *Plant Mol. Biol.*, **6**, 151−160.
Büschlen,S., Choquet,Y., Kuras,R. and Wollman,F.A. (1991) *FEBS Lett.*, **284**, 257−262.
Choquet,Y., Goldschmidt-Clermont,M., Girard-Bascou,J., Kück,U., Bennoun,P. and Rochaix,J.-D. (1988) *Cell*, **52**, 903−913.
Chua,N.-H. and Bennoun,P. (1975) *Proc. Natl Acad. Sci. USA*, **72**, 2175−2179.
de Vitry,C., Olive,J., Drapier,D., Recouvreur,M. and Wollman,F.-A. (1989) *J. Cell Biol.*, **109**, 991−1006.
Erickson,J.M. and Rochaix,J.-D. (1991) In Barber,J. (ed.), *Topics in Photosynthesis*. Elsevier Science Publishers, Amsterdam, Vol. 10, pp. 101−177.
Fenton,J.M. and Crofts,A.R. (1990) *Photosyn. Res.*, **26**, 59−66.
Goldschmidt-Clermont,M. (1991) *Nucleic Acids Res.*, **19**, 4083−4089.
Goldschmidt-Clermont,M., Girard-Bascou,J., Choquet,Y. and Rochaix,J.-D. (1990) *Mol. Gen. Genet.*, **223**, 417−425.

Goldschmidt-Clermont,M., Choquet,Y., Girard-Bascou,J., Michel,F., Schirmer-Rahire,M. and Rochaix,J.-D. (1991) *Cell*, **65**, 135–143.
Hallick,R.B., Hong,L., Drager,R.G., Faureau,M.R., Monfort,A., Orsat,B., Spielmann,A. and Stutz,E. (1993) *Nucleic Acids Res.*, **21**, 3537–3544.
Harris,E.H. (1989) *The Chlamydomonas Sourcebook. A Comprehensive Guide to Biology and Laboratory Use.* Academic Press, San Diego, CA.
Hiratsuka,J. *et al.* (1989) *Mol. Gen. Genet.*, **217**, 185–194.
Ikeuchi,M., Eggers,B., Shen,G., Webber,A., Yu,J., Hirano,A., Inoue,Y. and Vermaas,W. (1991) *J. Biol. Chem.*, **266**, 11111–11115.
Jensen,K.H., Herrin,D.L., Plumley,G. and Schmidt,G.W. (1986) *J. Cell Biol.*, **103**, 1315–1325.
Johnson,C.H. and Schmidt,G.W. (1993) *Plant Mol. Biol.*, **22**, 645–658.
Kuchka,M.R., Mayfield,S.P. and Rochaix,J.D. (1988) *EMBO J.*, **7**, 319–324.
Lemaire,C., Girard-Bascou,J., Wollman,F.A. and Bennoun,P. (1986) *Biochim. Biophys. Acta*, **851**, 229–238.
Mayfield,S.P., Rahire,M., Frank,G., Zuber,H. and Rochaix,J.D (1987) *Proc. Natl Acad. Sci. USA*, **84**, 749–753.
Monod,C., Goldschmidt-Clermont,M. and Rochaix,J.-D. (1992) *Mol. Gen. Genet.*, **231**, 449–459.
Ohyama,K. *et al.* (1986) *Nature*, **322**, 272–274.
Rochaix,J.-D., Kuchka,M., Mayfield,S.P., Schirmer-Rahire,M., Girard-Bascou,J. and Bennoun,P. (1989) *EMBO J.*, **8**, 1013–1021.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual.* 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schägger,H. and von Jagow,G. (1987) *Anal. Biochem.*, **166**, 369–379.
Shinozaki,K. *et al.* (1986) *EMBO J.*, **5**, 2043–2049.
Strasser,R.J. and Govindjee (1991) In Argyroudi-Akoyunoglou,J.H. (ed.), *Regulation of Chloroplast Biogenesis*. Plenum Press, New York, pp. 423–426.
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) *Methods Enzymol.*, **185**, 60–89.
Takahashi,Y., Goldschmidt-Clermont,M., Soen,S.-Y., Franzén,L.G. and Rochaix,J.-D. (1991) *EMBO J.*, **10**, 2033–2040.
Takahashi,Y., Matsumoto,H., Goldschmidt-Clermont,M. and Rochaix,J.D. (1994) *Plant Mol. Biol.*, in press.
Vermaas,W. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **44**, 457–481.
Weeks,D.P., Beerman,N. and Griffith,O. (1986) *Anal. Biochem.*, **152**, 376–385.
Zumbrunn,G., Schneider,M. and Rochaix,J.-D. (1989) *Technique*, **1**, 204–216.

Received on February 11, 1994; revised on March 31, 1994