The EMBO Journal Vol.16 No.20 pp.6095–6104, 1997

The chloroplast *ycf3* and *ycf4* open reading frames of *Chlamydomonas reinhardtii* are required for the accumulation of the photosystem I complex

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The chloroplast genes *ycf3* and *ycf4* from the green alga *Chlamydomonas reinhardtii* have been characterized. The deduced amino acid sequences of Ycf4 (197 residues) and Ycf3 (172 residues) display 41–52% and 64–78% sequence identity, respectively, with their homologues from algae, land plants and cyanobacteria. In *C.reinhardtii*, *ycf4* and *ycf3* are co-transcribed as members of the *rps9–ycf4–ycf3–rps18* polycistronic transcriptional unit into RNAs of 8.0 kb and 3.0 kb corresponding to the entire unit and to *rps9–ycf4–ycf3*, respectively. Using biolistic transformation, *ycf4* and *ycf3* were disrupted with a chloroplast selectable marker cassette. Transformants lacking *ycf4* or *ycf3* were unable to grow photoautotrophically and were deficient in photosystem I activity. Western blot analysis showed that the photosystem I (PSI) complex does not accumulate stably in thylakoid membranes of these transformants. Ycf4 and Ycf3 were localized on thylakoid membranes but not stably associated with the PSI complex and accumulated to wild-type levels in mutants lacking PSI. RNA blot hybridizations showed that transcripts of *psaA*, *psaB* and *psaC* accumulate normally in these mutants and use of chimeric reporter genes revealed that Ycf3 is not required for initiation of translation of *psaA* and *psaB* mRNA. Our results indicate that Ycf3 and Ycf4 are required for stable accumulation of the PSI complex.

Keywords: chloroplast genes/*C.reinhardtii*/open reading frame/photosystem I complex/thylakoid membrane

Introduction

The photosystem I complex (PSI) of the thylakoid membrane in cyanobacteria and chloroplasts from land plants and algae mediates the light-induced electron transfer from reduced plastocyanin or cytochrome c_6 to oxidized ferredoxin. In land plants and algae, PSI is a large multisubunit complex composed of at least five chloroplast-encoded subunits (PsaA, PsaB, PsaC, PsaI and PsaJ) and six nuclear-encoded subunits (PsaD, PsaE, PsaF, PsaG, PsaH and PsaK) (see Golbeck, 1992; Pakrasi, 1995). The PSI core consists of a heterodimer of the PsaA and PsaB subunits which bind the primary electron donor, P700, and the intermediate electron acceptors A_0, A_1 and F_X. The stromal PsaC subunit binds the terminal electron acceptors F_A and F_B, two [4Fe–4S] clusters. The PsaD and PsaE subunits, which are also located on the stromal side, have been shown to be involved in docking of ferredoxin to the PSI complex. In addition, PsaD is required for stable binding of PsaC to the reaction centre core. The lumenal PsaF subunit is involved in docking plastocyanin or cytochrome c_6 to the PSI complex and is required for efficient electron transfer from these reduced proteins to $P700^+$ (Hippler *et al.*, 1997). The functional roles of the remaining PSI subunits PsaI, PsaJ, PsaK and PsaM are presently unknown.

The biosynthesis of the PSI complex depends on the coordinated expression of nuclear and chloroplast genes, the targeting of the subunits to their proper location within the chloroplast, the association of the various redox cofactors, and the proper assembly of the subunits to form an active complex. To date, all identified factors required for the stable accumulation of the PSI complex are structural components of this complex. Factors involved specifically in transport or assembly of the PSI polypeptides have not been identified. The analysis of several PSI-deficient mutants of *Chlamydomonas reinhardtii* has revealed that failure to synthesize any of the PSI reaction centre polypeptides PsaA, PsaB or PsaC leads to the loss of PSI activity and increased turnover of the other PSI subunits (Girard-Bascou *et al.*, 1987; Takahashi *et al.*, 1991). Many nuclear PSI-deficient mutants analysed are affected in the maturation of the *psaA* transcript which originates from three separate exons and requires two *trans*-splicing events (see Rochaix, 1996). A nuclear PSI-deficient mutant has been recently reported to be affected at the level of translation of the PsaB subunit (Stampacchia *et al.*, 1997).

The complete nucleotide sequences of the chloroplast genomes from several land plants and algae have been determined (see Reardon and Price, 1995). The most conserved genes found among sequenced plastid genomes of photosynthetic organisms are involved in gene expression and photosynthesis. Several hypothetical chloroplast open reading frames (*ycf*) are conserved in algae, land plants and in the genome of the cyanobacterium *Synechocystis* sp. strain PCC 6803, suggesting that these genes are involved in some important function. To gain insight into the functional role of these unknown genes, chloroplast gene disruptions can be performed in the green alga *C.reinhardtii* using the chloroplast *aadA* expression cassette conferring spectinomycin resistance (Goldschmidt-Clermont, 1991) and biolistic transformation.

Here we have characterized the *C.reinhardtii ycf3* and *ycf4* genes which were previously mapped near each other

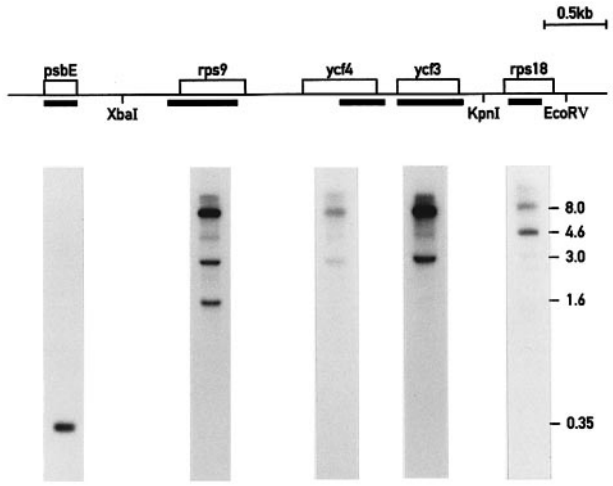

Fig. 1. Northern blot analysis of the *C.reinhardtii psbE, rps9, ycf4, ycf3* and *rps18*. Aliquots of total cellular RNA (5 μg) from wild-type cells were fractionated on denaturing 1.5% agarose–formaldehyde gel, blotted to a nylon membrane and hybridized with ^{32}P-labelled probes specific to *psbE, rps9, ycf4, ycf3* and *rps18*. The positions of the probes are indicated by horizontal lines below the gene map. The estimated sizes of RNA species are indicated in kb.

on the chloroplast genome (Boudreau *et al.*, 1994). These genes are expressed as part of the *rps9–ycf4–ycf3–rps18* polycistronic transcriptional unit and their products are associated with the thylakoid membrane. We show that *ycf3*- and *ycf4*-deficient mutants are unable to grow photoautotrophically and to accumulate PSI, in contrast to the cyanobacterial *ycf4*-deficient mutant in which PSI activity is only slightly reduced (Wilde *et al.*, 1995). It appears that Ycf3 and Ycf4 are not required for the synthesis of PSI subunits, but are most likely involved in the assembly and/or stability of the PSI complex. This is the first identification of chloroplast-encoded factors required for the accumulation of the PSI complex and acting at a post-translational level.

Results

Sequence analysis of the chloroplast DNA region containing ycf3 and ycf4

The *ycf3* and *ycf4* genes were previously mapped by Southern blot hybridization on the *C.reinhardtii* chloroplast *Pst*I fragment 4 and the *Eco*RI fragment R01 (Boudreau *et al.*, 1994). A *Eco*RV–*Xba*I subfragment from *Pst*I-4 which contains the R01 fragment and neighbouring DNA was entirely sequenced. The deduced 3595 bp nucleotide sequence revealed four open reading frames of 191, 197, 172 and 137 residues which were identified by DNA and amino acid sequence comparisons as *rps9, ycf4, ycf3* and *rps18*, respectively. The genes from the *rps9–ycf4–ycf3–rps18* cluster are all transcribed from the same DNA strand and the cluster lies 855 bp downstream of *psbE* which also has the same orientation (Figure 1). The *ycf4* and *ycf3* genes are separated by only 179 bp, *rps9* is located 401 bp upstream of *ycf4* and the beginning of *rps18* is 361 bp downstream of *ycf3*. The organization of *rps9, ycf4, ycf3* and *rps18* on the cpDNA is unique to *C.reinhardtii* since the corresponding genes on the chloroplast genome of land plants and other algae are dispersed and located on different DNA strands.

The *C.reinhardtii* Ycf3 and Ycf4 deduced amino acid sequences were compared by multiple sequence alignment with their homologues in land plants, algae and cyanobacteria (Figure 2A and B). The *C.reinhardtii* Ycf4 amino acid sequence exhibits significant sequence identity with the Ycf4 sequences from land plants (43.2–48.6%), the Euglenophyte *Euglena gracilis* (41.3%), the diatom *Odontella sinensis* (47.5%), the cyanelle of the Glaucocystophyte *Cyanophora paradoxa* (49.7%), the red alga *Porphyra purpurea* (52.2%) and the cyanobacterium *Synechocystis* sp. strain PCC 6803 (45.8%). Like its homologues (Wilde *et al.*, 1995), the *C.reinhardtii* Ycf4 contains two putative transmembrane α-helices within the N-terminal portion (Figure 2A). Its size is slightly larger than that of most previously described Ycf4 proteins, due to a 14 amino acid insertion between the two transmembrane domains. The Ycf3 protein from *C.reinhardtii* is more conserved than Ycf4 and shares greater sequence identity with its homologues from land plants (68.8–77.8%), the red algae *Porphyra purpurea* (69.8%) and *Cyanidium caldarium* (67.1%), the cyanelle of *Cyanophora paradoxa* (68.4%), the diatom *Odontella sinensis* (63.8%) and *Synechocystis* (67.8%).

Comparison of the *C.reinhardtii* Ycf3 amino acid sequence with protein databases revealed that different regions of the Ycf3 polypeptide display high homology with tetratricopeptide repeat motifs (TPR motifs) of *SPINDLY* in *Arabidopsis thaliana* (Jacobsen *et al.*, 1996). The TPR motif consists of a degenerate 34 amino acid consensus sequence which is often repeated in tandem arrays (Goebl and Yanagida, 1991). These motifs are found in numerous proteins of various functions and form amphipathic α-helices in two domains, the A-domain and the B-domain which are also referred to as the 'hole' and the 'knob', respectively. These domains are thought to be involved in protein–protein interactions (Lamb *et al.*, 1995). The *C.reinhardtii* Ycf3 sequence contains three putative TPR motifs (Figure 2C), two in tandem (position 35–105) followed by a third TPR sequence (position 106–153) split by a 14 amino acid spacer between the A-domain and the B-domain. The finding of TPR motifs within the Ycf3 sequences raises the possibility that this protein interacts with other proteins through this domain.

ycf4 and ycf3 are members of a large polycistronic transcriptional unit in C.reinhardtii

The transcriptional organization of the genes from the *rps9–ycf4–ycf3–rps18* cluster and *psbE* was analysed by hybridization of gene-specific probes to blots of total cellular RNA from *C.reinhardtii* separated by agarose–formaldehyde gel electrophoresis (Figure 1). The *ycf3* and *ycf4* gene-specific probes both hybridized to two major transcripts of 8.0 and 3.0 kb. The *rps9* gene probe also hybridized to the 8.0 and 3.0 kb RNAs in addition to a smaller transcript of 1.6 kb, whereas the *rps18* gene probe hybridized to two RNA species of 8.0 and 4.6 kb. Every gene-specific probe from the cluster detected an RNA with the same high molecular weight, suggesting that the *rps9–ycf4–ycf3–rps18* cluster is part of a polycistronic transcriptional unit that is transcribed into a very large RNA of 8.0 kb. The *psbE* gene probe only recognized one small transcript of 350 bases, as previously observed

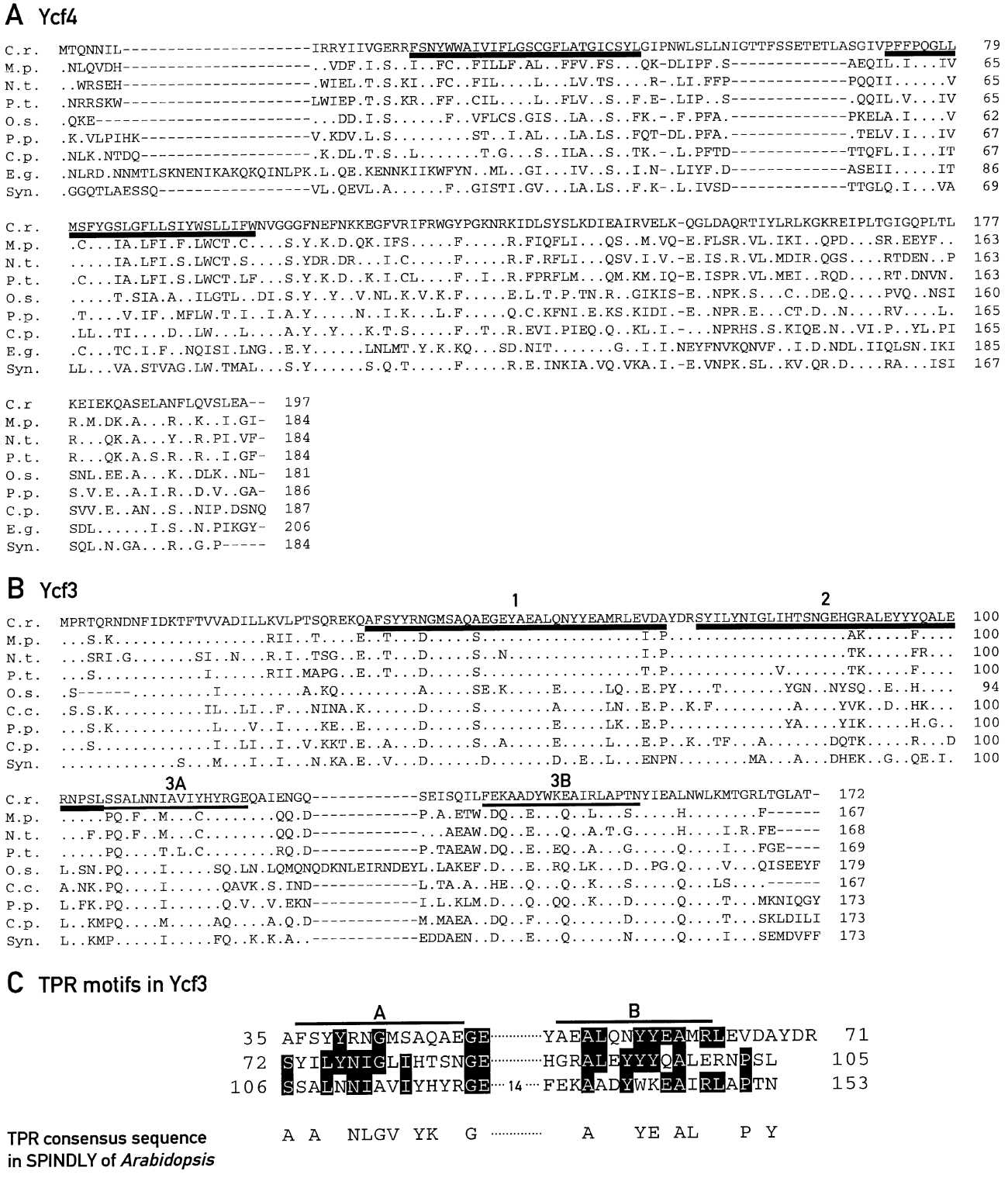

Fig. 2. Comparison of the deduced amino acid sequences of Ycf4 and Ycf3. (**A**) Sequence alignment of Ycf4 from *C.reinhardtii* (*C.r*), liverwort (*M.p*), tobacco (*N.t.*), black pine (*P.t.*), *Euglena gracilis* (*E.g.*), *Odontella sinensis* (*O.s.*), *Cyanophora paradoxa* (*C.p*), *Porphyra purpurea* (*P.p.*) and *Synechocystis* strain PCC 6803 (*Syn.*). The *C.reinhardtii* Ycf4 putative transmembrane helices are underlined. (**B**) Sequence alignment of Ycf3 from *C.reinhardtii* (*C.r*) with the corresponding ORFs from liverwort (*M.p*), tobacco (*N.t*), black pine (*P.t.*), *Odontella sinensis* (*O.s.*), *Cyanidium Caldarium* (*C.c*), *Porphyra purpurea* (*P.p.*), *Cyanophora paradoxa* (*C.p.*) and *Synechocystis* strain PCC 6803 (*Syn.*). The *C.reinhardtii* Ycf3 TPR motifs are underlined. (**C**) Alignment of the three *C.reinhardtii* Ycf3 TPR repeats with the TPR consensus motif observed in *SPINDLY* from *Arabidopsis thaliana* (Jacobsen *et al.*, 1996). The amino acids which are conserved in two of the three TPRs are boxed. The regions delimited by the TPR domains A and B are indicated over the alignment.

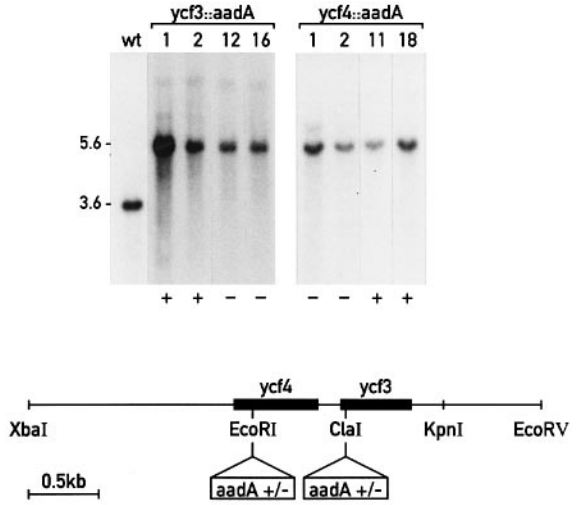

Fig. 3. Southern blot analysis of the *C.reinhardtii ycf3*::*aadA* and *ycf4*::*aadA* transformants. Total DNA from wild-type and transformants (designated by numbers) was digested with *Xba*I and *Eco*RV, electrophoresed, transferred to nylon membrane and hybridized with the *ycf4* gene-specific probe (see Figure 1). Wild-type (wt); four *ycf3*::*aadA* transformants (lanes 2–5 from the left); four *ycf4*::*aadA* transformants (lanes 6–9 from the left). The 2.0 kb *aadA* cassette was inserted into the unique *Eco*RI site of *ycf4* or the unique *Cla*I site of *ycf3*. The (+) and (–) indicate the transformants having the *aadA* cassette inserted in the same and opposite orientation, respectively, relative to the transcription of the genes. The estimated sizes of DNA fragments are indicated in kb.

(Mor *et al.*, 1995). Hence, *psbE* is probably not co-transcribed with the genes from the *rps9–ycf4–ycf3–rps18* transcription unit although it is oriented in the same way and close to this gene cluster. The 3.0 kb RNA to which the probes specific to *rps9*, *ycf4* and *ycf3* hybridized could be a primary transcript or it could result from the processing of the 8.0 kb transcript. The *rps9*, y*cf3* and *ycf4* probes hybridized faintly to a 4.8 kb RNA of unknown origin which is distinct from the 4.6 kb *rps18* transcript. This 4.8 kb signal appears to result from a cross-hybridization as it was also observed when either *ycf3* or *ycf4* was inactivated (data not shown, compare below). No transcripts corresponding to *ycf4* or *ycf3* monocistronic transcripts could be detected.

Disruption of ycf4 and ycf3

To gain insight into the functions of the Ycf3 and Ycf4 polypeptides, we disrupted their genes by means of biolistic transformation with the *aadA* expression cassette which confers spectinomycin resistance (Goldschmidt-Clermont, 1991). The *aadA* cassette was inserted in either orientation at the *Eco*RI site located 137 nucleotides downstream from the *ycf4* initiation codon or at the *Cla*I site positioned 34 nucleotides downstream from the *ycf3* initiation codon (Figure 3). Each of these constructs was used to transform a *C.reinhardtii* wild-type strain with a particle gun and the transformants were selected for their resistance to spectinomycin.

The *ycf3::aadA* and *ycf4::aadA* transformants were subjected to three rounds of single colony purification under selective conditions and their total cellular DNA was analysed by Southern blot hybridization (Figure 3). The DNA from wild-type and from the transformants was digested with *Xba*I and *Eco*RV and hybridized with the *ycf4* gene-specific probe. This probe hybridized with a 3.6 kbp fragment of wild-type DNA, whereas it hybridized exclusively with a 5.6 kbp DNA fragment from the *ycf3::aadA* and the *ycf4::aadA* transformants because the inserted cassette is 2 kbp long. The same 5.6 kbp fragment was detected when the blot was rehybridized with an *aadA* probe (data not shown). These results indicate that the *ycf3::aadA* and *ycf4::aadA* transformants are homoplasmic for the disrupted *ycf3* and *ycf4* genes, respectively.

The transcripts arising from the *ycf4–ycf3* gene cluster in the *ycf4*- and *ycf3*-deficient transformants with the *aadA* cassette oriented in both orientations were examined with probes specific for *rps9*, *ycf4*, *ycf3* and *rps18*. The results obtained are compatible with the existence of two major transcripts of 8 and 3 kb both of which start upstream of *rps9* (compare Figure 1 and data not shown).

Growth of the *ycf3::aadA* and *ycf4::aadA* transformants and wild-type strain was compared on acetate and minimal media under different light regimes. Both types of transformants are unable to grow photoautotrophically and their growth is severely impaired when these strains are grown mixotrophically under a light intensity of 80 $\mu E/m^2/s$ (data not shown), suggesting that Ycf3 and Ycf4 might be involved directly or indirectly in photosynthesis.

Loss of PSI complex in the ycf3::aadA and ycf4::aadA transformants

Measurements of fluorescence transients of several independently isolated dark-adapted *ycf3*- and *ycf4*-deficient transformants revealed a pattern characteristic of cells deficient in PSI or cytochrome b_6/f complex (Bennoun and Delepelaire, 1982). Rather than declining after reaching its maximum, as in wild-type cells, a continuous fluorescence rise was observed (data not shown).

In order to characterize the *C.reinhardtii ycf3* and *ycf4* products, antibodies were raised against recombinant proteins of Ycf3 and Ycf4 (for details, see Materials and methods). Whole cell proteins from wild-type and from the *ycf3::aadA* and *ycf4::aadA* transformants with the *aadA* cassette in both orientations were separated by PAGE, transferred to nitrocellulose membranes and probed with the Ycf3 and Ycf4 polyclonal antibodies. In the extracts from wild-type the Ycf3 and Ycf4 antisera detected polypeptides of 19 kDa and 22 kDa (Figure 4), respectively. As expected, the Ycf3 and Ycf4 proteins were not observed in the extracts from the transformants in which the corresponding gene had been disrupted. The Ycf4 protein was present in all *ycf3::aadA* transformants analysed. In contrast, the Ycf3 protein was detectable in the *ycf4::aadA* transformants with the *aadA* cassette inserted in the opposite direction relative to the operon, but not in those where the cassette had the other orientation. No *ycf3* transcript could be detected in the latter transformants by RNA blot analysis (data not shown). This is due to the fact that the 3′ *rbcL* transcription terminator/processing sequence on the *aadA* cassette (Goldschmidt-Clermont, 1991) prevents the accumulation of the transcript of the *ycf3* gene which is located downstream of *ycf4*.

To determine whether the loss of Ycf3 or Ycf4 had any effect on the accumulation of the PSI complex, whole cell extracts from wild-type, the *ycf3*- and *ycf4*-deficient strains

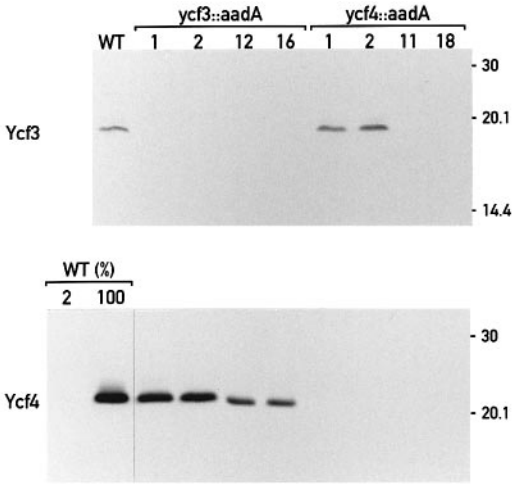

Fig. 4. Immunoblot analysis of Ycf3 and Ycf4 content in cells of wild-type and transformant strains. Total cell proteins (5 μg chlorophyll) of wild-type (WT), of the *ycf3*::*aadA* transformants (1 and 2) and the *ycf4*::*aadA* transformants (11 and 18) with the *aadA* cassette inserted in the same orientation as the *ycf4–ycf3* transcription unit, the *ycf3*::*aadA* transformants (12 and 16) and *ycf4*::*aadA* transformants (1 and 2) with the *aadA* cassette inserted in the opposite orientation. Molecular weight markers ($\times 10^3$) are indicated.

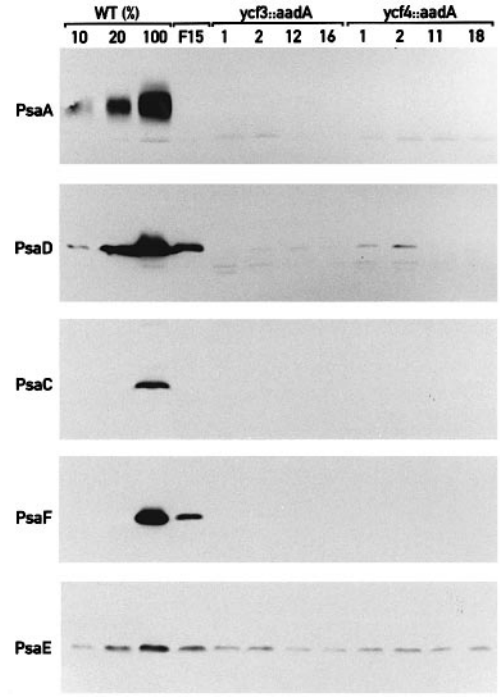

Fig. 5. Accumulation of PSI subunits in the *ycf3*- and *ycf4*-deficient strains. Immunoblot of thylakoid polypeptides (5 μg chlorophyll) separated by PAGE from wild-type (WT), the F15 mutant, the *ycf3*::*aadA* transformants (1, 2, 12 and 16) and *ycf4*::*aadA* transformants (1, 2, 11 and 18) probed with antisera against PsaA, PsaC, PsaD, PsaE and PsaF.

and F15, a mutant strain lacking PSI (Stampacchia *et al.*, 1997) were probed with antibodies directed against the PSI subunits PsaA, PsaD, PsaC, PsaF and PsaE. The immunoblot in Figure 5 shows that, with the exception of the PsaE subunit, the levels of all other PSI subunits are greatly reduced in the *ycf3*- and *ycf4*-deficient transformants. Even PsaD and PsaF, which accumulate to some

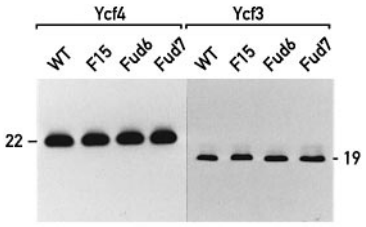

Fig. 6. Ycf3 and Ycf4 accumulate normally in mutants lacking photosynthetic complexes. Immunoblot of whole cell proteins fractionated by PAGE from wild-type (WT); F15, a photosystem I-deficient mutant; Fud6, a cytochrome b_6/f complex-deficient mutant and Fud7, a photosystem II-deficient mutant, probed with antisera against Ycf3 and Ycf4. The estimated sizes are indicated in kDa.

extent in the F15 mutant, are barely detectable in the transformants. Similar immunoblots with antibodies against the PSII reaction centre polypeptide D1, cytochrome *f*, the β-subunit of ATP synthase, LHCI and II proteins, revealed that these polypeptides accumulate to wild-type levels in the absence of either Ycf3 or Ycf4 and that the corresponding complexes are therefore not affected (data not shown).

Since the PSI complex no longer accumulates in the absence of Ycf3 or Ycf4, we investigated by Western analysis the accumulation of these proteins in mutants lacking PSI or other photosynthetic complexes. The immunoblot in Figure 6 shows that Ycf3 and Ycf4 accumulate normally in mutants lacking PSI (F15), PSII (Fud7) or the cytochrome b_6/f complex (Fud6) (Figure 6).

Absence of Ycf3 and Ycf4 does not affect the expression of PSI genes

The observed loss of the PSI complex induced by the absence of Ycf3 and Ycf4 raises the question of whether these proteins may be involved in the expression of the gene(s) of one or several PSI core subunits. To examine the expression of PSI genes at the transcriptional level, total cellular RNA from wild-type and from the *ycf3*- and *ycf4*-deficient strains was hybridized with PSI gene-specific probes for *psaA*, *psaB* and *psaC*. These hybridizations revealed that the level of these transcripts was the same in the mutants and in wild-type (data not shown).

To determine whether the loss of Ycf3 affects the expression of PSI subunits at the level of initiation of translation, we analysed the expression of the *psaA* 5′-UTR–*aadA* and *psaB* 5′-UTR–*aadA* chimeric reporter genes (Stampacchia *et al.*, 1997) in the absence of *ycf3*. The *ycf3* gene was disrupted with a recyclable *aadA* cassette (Fischer *et al.*, 1996) through biolistic transformation as described above. This recyclable *aadA* cassette is flanked by a 483 bp direct repeated sequence which undergoes homologous recombination and allows excision of the selectable marker when the selective pressure is relieved. After removal of the *aadA* cassette, the *ycf3*-deficient strain obtained was transformed with the chimeric *psaA* 5′-UTR–*aadA* gene by selecting for spectinomycin resistance. The transformants were able to grow in the presence of high concentrations of spectinomycin (500 μg/ml) (Figure 7).

The *ycf3* gene was also disrupted with the recyclable *aadA* cassette in a strain mutant for F15 and containing

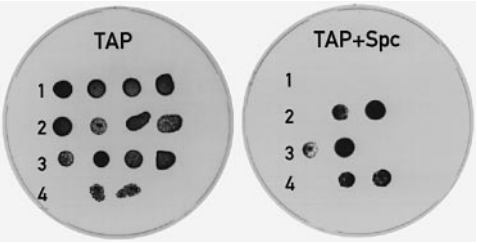

Fig. 7. Growth patterns of wild-type and the *ycf3*-deficient strains containing *psaA* 5′-UTR–*aadA* or *psaB* 5′-UTR–*aadA* chimeric genes in their chloroplast genome. Cells were spotted on either TAP medium (left) or TAP medium containing 500 μg/ml spectinomycin (right). First row from left to right: wild-type, F15 strain with *psaB* 5′-UTR–*aadA*, *ycf3*-deficient strain, *ycf3*-deficient F15 strain with *psaB* 5′-UTR–*aadA*. Second row: tetrad from the cross between the *ycf3*-deficient F15 strain containing *psaB* 5′-UTR–*aadA* (mt+) and wild-type (mt–). Third row: tetrad from the cross between the F15 strain containing *psaB* 5′-UTR–*aadA* (mt+) and wild-type (mt–). Fourth row: two independent, *ycf3*-deficient *psaA* 5′-UTR–*aadA* transformants.

the *psaB* 5′-UTR–*aadA* chimeric gene (Stampacchia *et al.*, 1997). This strain is spectinomycin-sensitive because the chimeric gene is not translated in the F15 nuclear background. The resulting *ycf3*-deficient F15 mutant strain containing the *psaB* 5′-UTR–*aadA* gene was crossed with a wild-type strain. All the progeny from this cross uniparentally inherited the inactivated *ycf3* gene and the chimeric *psaB* 5′-UTR–*aadA* reporter gene. Half of the progeny from this cross, containing the wild-type F15 allele, grew in the presence of spectinomycin (500 μg/ml) whereas the other half of the progeny, containing the mutant F15 allele, were unable to grow in the presence of the antibiotic (Figure 7). These results strongly suggest that Ycf3 is not required for the initiation of translation of the *psaA* and *psaB* mRNAs. Attempts to measure the rate of synthesis of PsaA and PsaB by pulse-labelling were inconclusive because these protein bands were too diffuse.

Immunolocalization of the Ycf3 and Ycf4 polypeptides

To determine the localization within the chloroplast of Ycf3 and Ycf4, wild-type cells from *C.reinhardtii* were broken and separated by centrifugation into a supernatant and pellet containing the membrane fraction. All of Ycf4 and most of Ycf3 were found in the pellet with a minor portion of Ycf3 in the supernatant (Figure 8A), suggesting that Ycf3 is loosely associated with the membranes. Fractionation of the membrane material by sucrose gradient centrifugation revealed that both Ycf3 and Ycf4 co-purify with the thylakoid membranes (Figure 8A). To examine how firmly Ycf3 and Ycf4 are associated with the thylakoid membranes, these were treated with salt and chaotropic agents known to wash out extrinsic proteins (Figure 8B). Ycf3 was completely released from the membranes after treatment with 0.1 M Na_2CO_3, pH 11.0 and partially removed with 2 M KSCN or 2 M KI. This protein remained associated with the membranes after treatment with 2 M NaCl or 2 M NaBr. Ycf4 was partially released from the membranes with Na_2CO_3 or KI and completely resistant to the other treatments (Figure 8B). These results suggest that Ycf3 and Ycf4 are not intrinsic membrane proteins and that Ycf4 is more tightly associated with the membranes than Ycf3. However, a transmembrane

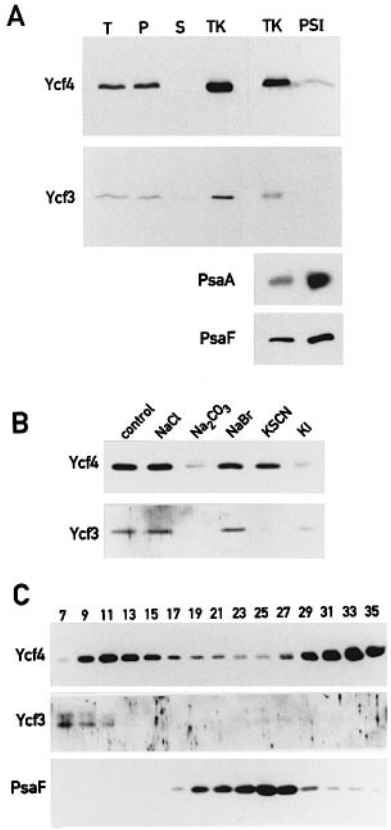

Fig. 8. Subcellular localization of Ycf3 and Ycf4 proteins examined by immunoblot analysis. (**A**) Western analysis of total cell proteins (T), the pellet containing the membrane fraction (P), the supernatant containing the soluble proteins (S), the thylakoid membrane proteins (TK) and PSI particles from wild-type. The blots were reacted with Ycf4, Ycf3, PsaA and PsaF antibodies. (**B**) Thylakoid membranes were incubated with 2 M NaCl, 0.1 M Na_2CO_3, 2 M KSCN and 2 M KI, centrifuged and the pellet was fractionated by PAGE, blotted on filters and reacted with Ycf4 and Ycf3 antisera. (**C**) Thylakoid membranes were solubilized with dodecyl–maltoside and sedimented on a sucrose density gradient at 240 000 *g* for 24 h at 4°C. 35 fractions were collected from the top of the gradient (on the left in the figure) and the proteins fractionated by PAGE, blotted and reacted with antibodies against Ycf4, Ycf3 and the PSI subunit PsaF.

orientation of Ycf4 cannot be ruled out since polypeptides with several transmembrane domains like cytochrome b_6 are released from the thylakoid membrane at high pH (Szczepaniak *et al.*, 1991).

Further fractionation of thylakoid membranes solubilized with dodecyl–maltoside on a continuous sucrose gradient by ultracentrifugation revealed that Ycf4 and Ycf3 do not co-fractionate with PSI in contrast to the authentic PSI subunits PsaA and PsaF (Figure 8A). A major portion of Ycf4 was found in the bottom fractions of the gradient (Figure 8C), suggesting that it may be part of a protein complex larger than PSI. Ycf4 could be loosely or transiently associated with this complex since part of Ycf4 was found also in the top and intermediate fractions of the gradient. The polypeptide profiles of the bottom fractions containing Ycf4 were examined by SDS–PAGE. However, it was not possible to identify polypeptides specifically associated with Ycf4. Under the same conditions Ycf3 was found mainly at the top of the gradient (Figure 8C, fractions 7–11) although trace amounts of

Ycf3 were also detected in fractions 25–29 which contain PSI.

The amounts of Ycf3 and Ycf4 protein were estimated relative to P700 using a dilution series of known amounts of recombinant Ycf4 and Ycf3 recombinant protein and a value of 905 chlorophyll molecules per P700 in thylakoid membranes of *C.reinhardtii* (Neale and Melis, 1986). Values of 1.2 Ycf4 and 0.03–0.06 Ycf3 per P700 were obtained. Thus, Ycf4 is present in stoichiometric amounts relative to PSI whereas the amount of Ycf3 protein is at least one order of magnitude lower.

Ycf3 and Ycf4 accumulation is reduced in the absence of chlorophyll

Since Ycf3 and Ycf4 are required for PSI accumulation, it was of interest to investigate whether the levels of these two factors are reduced under physiological conditions where PSI does not accumulate. This situation occurs in *y-1* mutant cells grown in the dark which are unable to synthesize chlorophyll and to accumulate the chlorophyll-containing PSI and PSII complexes and their associated light-harvesting systems. Upon illumination of dark-grown *y-1* cells, chlorophyll synthesis resumes followed by the gradual accumulation of PSI and PSII until they reach their wild-type levels after 8 h of light treatment (Ohad *et al.*, 1967; Malnoe *et al.*, 1988). It can be seen in Figure 9 that Ycf3 and Ycf4 in dark-grown *y-1* cells accumulate to 10–20% and 30–50% of the levels observed in light-grown *y-1* cells, respectively, and that the level of both proteins rises during illumination. It is noticeable that this rise precedes the appearance of the PSI subunit PsaA during the greening period.

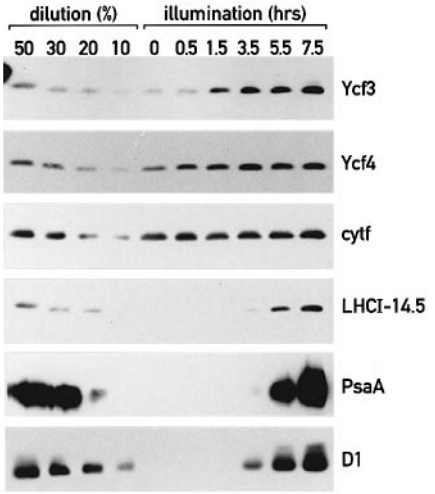

Fig. 9. Accumulation of Ycf3 and Ycf4 during the greening of the *y-1* mutant. The *y-1* mutant cells were grown in the dark for 5 days and transferred to light. Samples of the culture were collected at different times and total cell proteins were fractionated by PAGE and examined by immunoblotting with antibodies against Ycf3, Ycf4, cytochrome *f*, LHCI polypeptide 14.5, PsaA and D1 protein. A dilution series from the sample collected after 7.5 h of illumination was used to estimate the relative amount of protein accumulated.

Discussion

The ycf3 and ycf4 genes of C.reinhardtii are part of a large operon which includes the ribosomal protein genes rps9 and rps18

The *ycf3* and *ycf4* genes belong to transcription units of 8 and 3 kb. A remarkable feature is that a polycistronic transcript appears to be translated since no monocistronic transcript corresponding to either *ycf3* or *ycf4* could be detected. The organization of the genes from the *C.reinhardtii rps9–ycf4–ycf3–rps18* cluster differs extensively from that observed in other organisms. In land plants, *ycf4*, *ycf3* and *rps18* map to different clusters (*psaI–ycf4–ycf10–petA*, *ycf3–psaA–psaB–rps14* and *rps18–rpl33*) located on different DNA strands of the chloroplast genome (Palmer, 1991) whereas *rps9* has not been found in the land plant cpDNA. However, this gene is present in the chloroplast genome of non-green algae (Reardon and Price, 1995). It may have been transferred to the nuclear genome of land plants as proposed for other algal chloroplast genes such as *tufA* and *rpl22* (Baldauf *et al.*, 1990; Gantt *et al.*, 1991).

It is remarkable that *ycf4* and *ycf3* are the only genes from the *C.reinhardtii rps9–ycf4–ycf3–rps18* cluster that have remained linked together in the chloroplast genome of different representatives of the two major lineages of the polyphyletic genus *Chlamydomonas* (Boudreau *et al.*, 1994; Boudreau and Turmel, 1995, 1996). Multiple sequence rearrangements marked the evolution of the *Chlamydomonas* chloroplast genome and only a few clusters have been found to be conserved in *C.reinhardtii* and *Chlamydomonas moewusii* (Boudreau *et al.*, 1994), the representatives of the two major *Chlamydomonas* lineages (Buchheim *et al.*, 1996). Many chloroplast genes that are tightly linked and co-transcribed in *C.reinhardtii* are dispersed loci in *C.moewusii*. The observation that *ycf4* and *ycf3* are close to each other in every *Chlamydomonas* lineage analysed suggests that these genes were most probably linked together in the most recent common ancestor of all *Chlamydomonas* species.

Ycf4 and Ycf3 are extrinsic thylakoid membrane polypeptides required for the accumulation of the PSI complex in C.reinhardtii

This study has shown that inactivation of *ycf3* or *ycf4* in *C.reinhardtii* leads in both cases to a deficiency in PSI activity and to the inability of the mutant cells to grow photoautotrophically. A striking feature in these mutants is that the accumulation of the PSI subunits is nearly undetectable, even though Ycf3 and Ycf4 do not appear to be associated stably with PSI. However, it is possible that Ycf3 and Ycf4 interact loosely with PSI and that this interaction is easily disrupted by treatments with detergent. The loss of PSI in the *ycf3*- and *ycf4*-deficient mutants appears to be specific as the level of the other thylakoid protein complexes (PSII, the cytochrome b_6/f complex, ATP synthase and the LHC complex) are unaffected in these mutants. Furthermore, both proteins accumulate to wild-type levels in mutants lacking PSI, PSII or the cytochrome b_6/f complex.

The immunoblot data indicate that both Ycf3 and Ycf4 are associated with the thylakoid membrane. The sequence of Ycf4 reveals two hydrophobic regions which could act as potential transmembrane helices. However, treatment of the thylakoid membranes with alkali or some chaotropic agents showed that both Ycf3 and Ycf4 are extrinsic membrane proteins.

One possibility is that these polypeptides are required for the synthesis of one or several PSI core subunits, as it is well documented that loss of any of these subunits destabilizes the PSI complex (Girard-Bascou *et al.*, 1987; Takahashi *et al.*, 1991; Stampacchia *et al.*, 1997). Some tertiary structure similarity has been noticed between the 100 carboxy-terminal residues of the Ycf3 protein and the homeodomain of transcription factors (Yura and Go, 1997). A role of Ycf3 and Ycf4 in chloroplast transcription can, however, be ruled out since the levels of the *psaA*, *psaB* and *psaC* transcripts were unaffected in the *ycf3*- and *ycf4*-deficient mutants. Further, Ycf3 is not required for the initiation of translation of *psaA* and *psaB* mRNA since the expression of chimeric genes driven by the *psaA* and *psaB* 5′-UTRs was not diminished in the *ycf3*-deficient strains. A role of Ycf3 and Ycf4 in *psaC* mRNA translation is unlikely since the amount of PSI complex which accumulates in the absence of PsaC (Takahashi *et al.*, 1991) is significantly higher than in the absence of either Ycf3 or Ycf4. While we cannot completely rule out the possibility that Ycf3 may be required specifically for translation elongation of *psaA* and *psaB* mRNA, it is rather unlikely that Ycf4 plays any role in translation, since inactivation of *ycf4* in *Synechocystis* does not alter the growth rate under photoautotrophic conditions and the PSI complex still accumulates, albeit in reduced amount (Wilde *et al.*, 1995). It is highly unlikely that the loss of PSI in the *ycf3*- and *ycf4*-deficient strains is mediated through *psaI* and *psaJ*. First, disruption of *psaJ* does not affect photoautotrophic growth of *C.reinhardtii* (N.Fischer, E.Boudreau and J.D.Rochaix, unpublished results). Second, disruption of *psaI* in cyanobacteria does not prevent photoautotrophic growth (Xu *et al.*, 1995).

Another possibility is that Ycf3 and Ycf4 are required for the assembly and/or stability of the PSI complex. It is apparent that the absence of Ycf4 has a stronger effect in *C.reinhardtii* than in cyanobacteria. Whereas the PSI complex is fully destabilized in the algal mutant, it is still functional in the corresponding cyanobacterial mutant (Wilde *et al.*, 1995). The higher PSII/PSI ratio observed in this mutant was attributed to an increase in the level of PSII complex and to a slight reduction in the amount of PSI and a role of Ycf4 in the structural organization of the photosynthetic membrane was proposed (Wilde *et al.*, 1995). Similar phenotypic differences between *C.reinhardtii* and cyanobacteria have been observed for other inactivations of genes involved in photosynthesis. The absence of the PsaC subunit leads to an almost complete loss of the PSI complex (Takahashi *et al.*, 1991), whereas in cyanobacteria the PSI reaction centre can assemble in the absence of PsaC, although it is deficient in photochemical activity (Mannan *et al.*, 1991; Yu *et al.*, 1995). Similarly, the loss of the PsbK and the PsbO subunits of the PSII complex in *C.reinhardtii* leads to the destabilization of the PSII complex (Mayfield *et al.*, 1987; Takahashi *et al.*, 1994), whereas in cyanobacteria these mutants grow photoautotrophically, although at reduced rates (Burnap and Sherman, 1991; Ikeuchi *et al.*, 1991). These observations raise the possibility that *C.reinhardtii* cells possess a chloroplast 'clearing system' that recognizes and degrades polypeptides of misassembled protein complexes which is either not present or not as efficient in cyanobacteria.

The amount of Ycf3 protein is at least one order of magnitude lower than that of PSI. This polypeptide is the first chloroplast-encoded protein identified that contains TPR motifs within its sequence. The presence of these motifs suggests that Ycf3 may interact with other proteins and/or that it might be part of a protein complex, although no stable complex could be detected. The biogenesis of the PSI complex requires the targeting of numerous hydrophobic and hydrophilic subunits of chloroplast and nuclear origin to the PSI assembly site in the thylakoid membrane and the proper assembly of these polypeptides into a functional complex. Several chloroplast proteins were shown to interact with the chloroplast chaperones Cpn60 and Hsp70 (Lubben *et al.*, 1989; Madueño *et al.*, 1993). These chaperones are believed to assist the chloroplast proteins in maintaining the proper conformation required for correct assembly and localization. It is conceivable that Ycf3 may interact with free PSI subunits and is involved in folding, insertion and/or assembly of the PSI proteins in the thylakoid membrane, in an analogous manner to chaperones. In this regard, it is interesting to note that the level of Ycf3 in the *y-1* mutant increases upon illumination of dark-adapted cells and that this increase precedes the appearance of the chloroplast-encoded PSI subunits during the greening process.

The fact that Ycf4 and Ycf3 are not associated with the isolated PSI complex suggests that these proteins are not required for PSI stability, at least not *in vitro*. The Ycf4 protein is present in stoichiometric amounts relative to PSI. It is possible that this protein is required for the proper insertion of the PSI complex within the thylakoid membrane. Ycf4 may be part of a large protein complex which remains to be characterized (see Figure 8C).

The PSI complex is known to contain several redox cofactors including the chlorophyll dimer P700, the primary electron acceptors A_0 and A_1 and the 4Fe–4S clusters F_X, F_A and F_B. Little is known about how these cofactors are inserted into the PSI complex and whether specific factors are required. A role of Ycf3 in these processes cannot be excluded. It has been shown recently that the chloroplast *ycf5* gene is required for heme attachment to chloroplast *c*-type cytochromes (Xie and Merchant, 1996).

The Ycf3 and Ycf4 factors have properties similar to some of the factors identified in yeast mitochondria which are required for the assembly of various respiratory complexes such as cytochrome oxidase, ubiquinol cytochrome *c* reductase and ATP synthase (Tzagoloff *et al.*, 1994; Glerum *et al.*, 1995; Altamura *et al.*, 1996). Most of these factors have been shown to be embedded in the mitochondrial inner membrane and some are associated with a high-molecular weight complex. Several of these factors are members of the ATP-dependent AAA-family (ATPases associated with a variety of cellular activities) that are involved both in the assembly and degradation of mitochondrial membrane protein complexes (Leonhard *et al.*, 1996). However, no ATPase domain could be identified in Ycf3 and Ycf4.

Materials and methods

DNA sequencing and analysis

Double-stranded DNA templates were sequenced with synthetic oligonucleotides using the dideoxy chain termination method with the T7

sequencing kit from Pharmacia Biotech Inc. (Piscataway, NJ) or the PRISM™ Ready Reaction Dye Deoxy Termination Cycle Sequencing Kit from Applied Biosystem Inc (Foster City, CA). Sequence analysis was performed using the Genetics Computer Group Software (Version 8, Genetics Computer Group, University of Wisconsin, Madison, WI).

DNA constructs

A 2.9 kb *Kpn*I–*Xba*I fragment from the *C.reinhardtii Pst*I fragment 4 containing *ycf3* and *ycf4* (see Figure 1) was subcloned in the plasmid vector pBluescript KS^+ (Stratagene, La Jolla, CA). The resulting recombinant plasmid was digested with either *Cla*I or *Eco*RI and the linearized plasmids were subsequently blunted by treating with T4 DNA polymerase. The 2.0 kb *aadA* expression cassette conferring spectinomycin resistance was excised from the plasmid pUC–atpX–AAD (Goldschmidt-Clermont, 1991) by digesting with *Eco*RV and *Sma*I and ligated to the *Cla*I or *Eco*RI linearized plasmids to inactivate *ycf3* and *ycf4*, respectively. The 3.0 kb recyclable *aadA* cassette was excised from the plasmid pKS-483–AAD-483 (Fischer *et al.*, 1996) by digesting with *Kpn*I and *Sac*I, blunted with T4 DNA polymerase and ligated to the *Cla*I linearized plasmid in order to inactivate *ycf3*. DNA manipulations and cloning were carried out using standard procedures (Sambrook *et al.*, 1989).

Transformation and genetic analysis

Chloroplast transformations of a *C.reinhardtii* wild-type strain with the *ycf3*::*aadA* (+/–), *ycf4*::*aadA* (+/–) and *ycf3*::483–*aadA*-483 constructs and of the F15 mutant strain containing the *psaB* 5′-UTR–*aadA* reporter gene (Stampacchia *et al.*, 1997) with *ycf3*::483–*aadA*-483 were performed as described previously (Goldschmidt-Clermont, 1991). Transformants were selected on TAP agar plates containing 150 μg/ml spectinomycin and recloned three times on TAP–spectinomycin plates. The *aadA* recyclable cassette was removed from the *ycf3*::483–*aadA*-483 transformants as described by Fischer *et al.* (1996). The *ycf3*-disrupted strain was transformed with the *psaA* 5′-UTR–*aadA* chimeric gene (Stampacchia *et al.*, 1997). The F15 mutant strain containing the *psaB* 5′-UTR–*aadA* chimeric gene and a deletion of *ycf3* [mating type (+)] was crossed to a wild-type strain [mating type (–)]. The resulting transformants and progeny were tested for growth on TAP agar plates containing 500 μg/ml spectinomycin. Crosses were carried out according to Harris (1989).

Isolation of nucleic acids and hybridizations

Total DNA and RNA from wild-type and transformants were extracted as previously described (Boudreau *et al.*, 1997). The DNA preparations were double digested with *Eco*RV and *Xba*I restriction endonucleases. The resulting fragments were separated by agarose gel electrophoresis, transferred onto Hybond–Nylon membranes (Amersham, Arlington Heights, IL) and hybridized with a ^{32}P-labelled 368 bp PCR-amplified fragment specific for *ycf4* (positions +299, ++81 relative to the start of the ORF; + and – refer to nucleotides downstream and upstream of the initiation codon, respectively; ++ refers to nucleotides downstream of the stop codon) and ^{32}P-labelled 2.0 kb *Eco*RV–*Sma*I fragment containing the *aadA* cassette. RNA aliquots (5 μg) were electrophoresed and transferred to Nytran nylon membranes (Schleicher and Schuell, Keene, NH) and hybridized with ^{32}P-labelled PCR-amplified probes that were specific for *psbE* (268 bp, –11,++8), *rps9* (576 bp, positions –75, +501), *rps18* (277 bp, positions +35, +311), *ycf3* (565 bp, positions –16, ++31) and *ycf4*.

Antiserum production

The *ycf3* open reading frame was amplified by PCR using a pair of synthetic oligonucleotides: 5′-GCTGATTTTATTTCATATGCCAAGA-ACGCA-3′ and 5′-CAAGTTCAAGTCGACAGGGTCAACATCTT-3′. *Nde*I and *Sal*I restriction sites were introduced within the 5′ and 3′ *ycf3* oligonucleotides, respectively. The 3′ half of the *ycf4* open reading frame (positions +299, ++81) was amplified by PCR using a pair of synthetic oligonucleotides: 5′-CGGCAGGCATATGGGTGGTGGTTTTAATGAA-3′ and 5′-GCGCCAAAGGTGGAGCTCAATAACATCTGT-3′. *Nde*I and *Sac*I restriction sites were introduced within the 5′ and 3′ *ycf4* oligonucleotides, respectively. The *ycf3* and *ycf4* amplified DNA fragments were digested with *Nde*I–*Sal*I and *Nde*I–*Sac*I, respectively, and cloned into the corresponding sites of the pET28a expression vector (Novagen Inc., Madison, WI). Constructs were introduced into the expression host *Escherichia coli* strain BL21 by transformation. Expression was induced with 1 mM isopropylthio-β-D-galactoside. The recombinant proteins were purified with the pET HIS–Tag System (Novagen) in denaturing conditions. Purified recombinant protein (70 μg) was mixed with Freund's adjuvant and injected subcutaneously into rabbits. Six booster injections were performed until the antibody titre had reached its maximum.

Immunoblot analysis

Cells from *ycf3*- and *ycf4*-deficient transformants and wild-type were grown in TAP medium under dim light at 25°C ($2–4\times10^6$ cells/ml). Thylakoid membranes were prepared as described by Chua and Bennoun (1975). To examine the association of Ycf3 and Ycf4 with the thylakoid membranes (80 μg in 200 μl), they were treated with 2 M NaCl, 2 M NaBr, 2 M KSCN, 2 M KI or 0.1 M Na_2CO_3, pH 11.0 for 10 min at room temperature, diluted 5-fold with H_2O and pelletted by centrifugation. The washed membranes were resuspended in 25% glycerol. Chlorophyll–protein complexes were solubilized with 0.05% *n*-dodecyl-β-D-maltoside from wild-type thylakoids and separated by centrifugation on a 0.1–1.0 M linear sucrose density gradient at 240 000 *g* for 24 h as described (Takahashi *et al.*, 1991). Proteins were separated by SDS–PAGE (Laemmli, 1970) with 15% acrylamide and 12.5% glycerol in the resolving gel. Proteins were blotted onto nitrocellulose filters, reacted with antisera and the signals were visualized by enhanced chemiluminescence (ECL) method.

Greening of *y-1* mutant cells

The *y-1* mutant cells were grown in the dark at 25°C to $4–6\times10^6$ cells/ml and then exposed to light. Total cell proteins equivalent to 3×10^6 cells were subjected to immunoblot analysis.

Estimation of the amount of Ycf3 and Ycf4 proteins in cells

The concentrations of purified Ycf3 and Ycf4 recombinant proteins were estimated with the bicinchoninic acid (BCA) assay reagent (Pierce). For quantitative estimation of the Ycf3 and Ycf4 proteins in the wild-type cells (5 μg chlorophyll), known amounts of recombinant Ycf3 or Ycf4 protein were mixed with extracts from *ycf3*- or *ycf4*-deficient cells (5 μg chlorophyll), respectively, and the mixtures were subjected to immunoblot analysis. Signals obtained with the recombinant proteins were compared with those obtained with authentic proteins in wild-type cell extracts.

Accession number

The accession number of the ycf3-ycf4 region is Y13655.

Acknowledgements

We thank M.Goldschmidt-Clermont, M.Hippler, K.Redding and O.Stampacchia for helpful comments. This work was supported by a grant from the Human Frontier Science programme and by grant 31-34014.92 from the Swiss National Fund to J.D.R., by a grant for Scientific Research from the Ministry of Education, Science and Culture, Japan to Y.T., by grants from the Natural Sciences and Engineering Research Council of Canada and from 'Le Fonds pour la Formation de Chercheurs et l'Aide à la Recherche' to M.T. and C.L.; M.T. and C.L. are Scholars in the Evolutionary Biology Program of the Canadian Institute for Advanced Research (CIAR) and are grateful for salary support from CIAR. E.B. was supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada.

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Received on June 9, 1997; revised on July 18, 1997