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–eps18* 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* 5 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, PsaI 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 luminal PsaF subunit is involved in docking plastocyanin or cytochrome *c* 5 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...
on the chloroplast genome (Boudreau et al., 1994). These
genotypes 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 photo-
autotrophically and to accumulate PSI, in contrast to the
cyanoalgal 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 chloro-
plast PsII fragment 4 and the EcoRI fragment R01
(Boudreau et al., 1994). An EcoRV–XbaI subfragment from
PsII-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 cyanobac-
teria (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 Ondon-
tella sinensis (47.5%), the cyanelle of the Glaucocysto-
phyte 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 Ondotella 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 (Goebel 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 the 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* (Cr), liverwort (M.p.), black pine (Pt.), tobacco (T.), Euglena gracilis (E.g.), Odontella sinensis (O.s.), *Cyanidium* caldarium (C.c.), *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* (Cr) with the corresponding ORFs from liverwort (M.p.), black pine (Pt.), *Porphyra* purpurea (P.p.), *Cyanidium* caldarium (C.c.) and *Synechocystis* strain PCC 6803 (Syn.). The *C. reinhardtii* Ycf3 TPR motifs are underlined.
The 2.0 kb cassette inserted in the same and opposite orientation, respectively, ycf3 inserted into the unique ycf3 transformants (designated by numbers) was digested with Xba. ycf4 with the Eco ycf4 used to transform a initiation codon (Figure 3). Each of these constructs was ycf3 site positioned 34 nucleotides downstream from the Xba. The DNA from wild-type and from the transformants was under selective conditions and their total cellular DNA was analysed by Southern blot hybridization (Figure 3).

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 µE/m²/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/f complex (Benoun 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...
and Ycf4 are required for PSI accumulation

**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 (×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, PsaD, PsaC, PsaE and PsaF.

**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 b6/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.

**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 extent in the F15 mutant, are barely detectable in the transformants. Similar immunoblots with antibodies against the PSI reaction centre polypeptide D1, cytochrome f, the β-subunit of ATP synthase, LHCl 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 b6/f complex (Fud6) (Figure 6).
the \textit{psaB 5'}-\textit{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 \textit{ycf3}-deficient F15 mutant strain containing the \textit{psaB 5'}-\textit{UTR--aadA} gene was crossed with a wild-type strain. All the progeny from this cross unparentally inherited the \textit{ycf3} gene and the chimeric \textit{psaB 5'}-\textit{UTR--aadA} reporter gene. Half of the progeny from this cross, containing the wild-type F15 allele, grew in the presence of spectinomycin (500 \(\mu\)g/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 \textit{Ycf3} is not required for the initiation of translation of the \textit{psaA} and \textit{psaB} mRNAs. Attempts to measure the rate of synthesis of \textit{PsaA} and \textit{PsaB} by pulse-labelling were inconclusive because these protein bands were too diffuse.

\textbf{Immunolocalization of the Ycf3 and Ycf4 polypeptides}

To determine the localization within the chloroplast of Ycf3 and Ycf4, wild-type cells from \textit{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\textsubscript{2}CO\textsubscript{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\textsubscript{2}CO\textsubscript{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 orientation of Ycf4 cannot be ruled out since polypeptides with several transmembrane domains like cytochrome \textit{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.

**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 (psal–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 phototrophically. 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 b6/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 b6/f complex.

The immunoblot data indicate 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 psaA and psaI. First, disruption of psaA 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 A0 and A1 and the 4Fe-4S clusters FX, FA and FB. 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
DNA constructs

A 2.9 kb KpnI-XbaI fragment from the *Chlorella variabilis* plastid fragment 4 containing ycf3 and ycf4 (see Figure 1) was subcloned in the plasmid vector pHBluscript KS+ (Strategene, La Jolla, CA). The resulting recombinant plasmid was digested with either ClaI or EcoRI 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-aptX–AAD (Goldschmidt-Clermont, 1991) by digesting with EcoRV and SmaI and ligated to the ClaI or EcoRI 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 KpnI and SacI, blunted with T4 DNA polymerase and ligated to the ClaI 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 *Chlorella variabilis* wild-type strain with the ycf3::aadA (+/+), ycf4::aadA (+/−) and ycf3:483–aadA–483 constructs and of the F15 mutant strain containing the psaA 5′-UTR-aadA 4′UTR reporter gene (Stampacchia et al., 1997) with ycf3:483–aadA–483 4′UTR was performed and 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 4′UTR transforms 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 ycf5 [mating type (+)] was crossed to a wild-type strain [mating type (−)]. The resulting recombinants 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 EcoRV and XbaI restriction endonucleases. The resulting fragments were separated by agarose gel electrophoresis, transferred onto Hybond-Nyl membranes (Amersham, Arlington Heights, IL) and hybridized with a 32P-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 32P-labelled 2.0 kb EcoRV-SmaI fragment containing the ycf3 or ycf4 open reading frames. RNA aliquots (5 μg) were electrophoresed and transferred to Nytran nylon membranes (Schleicher and Schuell, Keene, NH) and hybridized with 32P-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′-GGTCTTTTATTTCTATGCCAAGAACGCA-3′ and 5′-CAATGCAATGTCAGGCTAACATCTTG-3′. NdeI and SalI restriction sites were introduced within the 5′ and 3′ ycf3 oligonucleotides, respectively. The 3′ half of the ycf3 open reading frame (positions +299, +81) was amplified by PCR using a pair of synthetic oligonucleotides: 5′-CGCGAGCATATGGGGTGGTTATTTAGGAAGCT-3′ and 5′-GGCCCAAGTGTTGGGATCTAATACCTCTTG-3′. NdeI and SacI restriction sites were introduced within the 5′ and 3′ ycf4 oligonucleotides, respectively. The ycf3 and ycf4 amplified DNA fragments were digested with NdeI-SalI and NdeI-SacI, 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 (2–4×10⁶ 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 (800 μg in 200 μl), they were treated with 2 M NaCl, 2 M NaBr, 2 M KSCN, 2 M KI or 0.1 M NaCO₃, pH 11.0 for 10 min at room temperature, diluted 5-fold with H₂O and pelleted by centrifugation. The washed membranes were resuspended in 25% glycerol. Chlorophyll–protein complexes were solubilized with 0.05% 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 4–6×10⁹ cells/ml and then exposed to light. Total cell proteins equivalent to 3×10⁶ 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.

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References


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