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Initiation codon mutations in the *Chlamydomonas* chloroplast *petD* gene result in temperature-sensitive photosynthetic growth

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The chloroplast *petD* gene encodes subunit IV of the cytochrome b_6/f complex and is required for photosynthetic electron transport. We have created *Chlamydomonas* strains in which the initiation codon of the *petD* gene has been changed to AUU or AUC. These mutants can grow photosynthetically at room temperature, but not at 35°C. The accumulation of subunit IV during photosynthetic or heterotrophic growth at room temperature is reduced to 10–20% of the wild-type level; *petD* mRNA abundance is reduced to ~50% of the wild-type amount. Pulse labeling experiments indicate that at room temperature, subunit IV translation proceeds at 10–20% of the wild-type rate. Cells grown heterotrophically at 35°C accumulate <5% as much subunit IV as wild-type cells grown under the same conditions, and <1% as much subunit IV as wild-type cells grown at room temperature. We conclude that translation initiation in these mutants is inefficient, leading to decreased translation and accumulation of subunit IV. At 35°C, translational inefficiency leads directly or indirectly to insufficient accumulation of subunit IV to support photosynthetic growth.

Key words: *Chlamydomonas*/chloroplast mutant/chloroplast transformation/translation initiation

Introduction

Molecular, genetic and biochemical studies indicate that the chloroplast translational apparatus resembles that of prokaryotes. Genes encoding the chloroplast counterparts of many components of the *Escherichia coli* translational apparatus have been found in nuclear and chloroplast DNAs, for example 16S, 23S and 5S rRNAs, tRNAs, ribosomal proteins, elongation factor EF-Tu (*tufA*) and initiation factor IF-1 (*infA*) (Suguira, 1992). In addition, it has been shown that *Euglena* chloroplast translation initiation factors can, to some extent, substitute for their *E.coli* counterparts *in vitro* (Gold and Spremulli, 1985; Kraus and Spremulli, 1986). Finally, chloroplast ribosomes are similar to prokaryotic ribosomes, i.e. they sediment at 70S, with 30S and 50S subunits, and are sensitive to translational inhibitors such as chloramphenicol, but are resistant to inhibitors of 80S ribosomes, such as cycloheximide.

A critical phase of translation initiation is the selection of the correct start codon, which for most mRNAs in *E.coli* and chloroplasts is AUG; GUG accounts for most of the exceptions (Hershey, 1987). In *E.coli*, correct initiation is facilitated by the interaction of the 3′ end of 16S rRNA with the Shine–Dalgarno sequence in the 5′ untranslated region (5′ UTR) of the mRNA (Gold *et al.*, 1981), and in some cases, also by the interaction of ribosomal protein S1 with a pyrimidine-rich sequence in the 5′ UTR (Boni *et al.*, 1991). The spinach chloroplast homolog of S1 has also been shown to bind the 5′ UTR of ribosomal protein L22 mRNA *in vitro* (Franzetti *et al.*, 1992).

In spite of the conservation of core translational components between chloroplasts and *E.coli*, translation initiation in chloroplasts has several unusual features that distinguish it from both bacterial and cytosolic translation. First, although a ribosome binding site similar to the Shine–Dalgarno sequence has been proposed based on comparisons of 5′ UTR sequences from chloroplast genes of various plants (Ruf and Koessel, 1988; Bonham-Smith and Bourque, 1989), this proposed ribosome binding site is not found at a fixed distance from the initiation codon, even within the same plant. In contrast, the position of the Shine–Dalgarno sequence is fixed in *E.coli*. Second, although both higher plant chloroplasts and *E.coli* synthesize polycistronic primary transcripts, in chloroplasts these transcripts are processed extensively by endonucleolytic and exonucleolytic pathways (Hudson *et al.*, 1987; Rock *et al.*, 1987; Westhoff and Herrmann, 1988). Both polycistronic and monocistronic transcripts are functional mRNAs in chloroplasts (Barkan, 1988), while in bacteria, most translated messages are polycistronic. In *Chlamydomonas*, nearly all chloroplast genes appear to be transcribed and translated as monocistronic mRNAs.

Finally, mechanisms have evolved in chloroplasts to regulate translation of specific mRNAs, sometimes in response to light and developmental signals. *Cis*-acting elements that control translation have been identified in the 5′ UTRs of the tobacco *psbA* and *Chlamydomonas petD* mRNAs by introducing chimeric genes into the corresponding chloroplast genome (Sakamoto *et al.*, 1993; Staub and Maliga, 1993). Evidence for nuclear genes involved in the translation of specific chloroplast genes comes from both genetic and biochemical studies. For example, nuclear mutations that affect translation of the photosystem II chlorophyll apoprotein CP43 in *Chlamydomonas* are partially suppressed by a chloroplast mutation that maps within a potential stem–loop structure in the 5′ UTR of *psbC* mRNA (Rochaix *et al.*, 1989). Furthermore, an RNA binding protein that interacts with the *psbA* 5′ UTR has been identified, and its abundance correlates with the rate of *psbA* translation (Danon and Mayfield, 1991). Translation of chloroplast mRNAs thus appears to be regulated at multiple steps in a manner distinct from that of prokaryotes.

We have elected to use a molecular genetic approach to study translation initiation in chloroplasts, using the eukaryotic unicellular green alga *Chlamydomonas reinhardtii* as a model system. Because non-photosynthetic mutants can be maintained by growth on a reduced carbon source,

Chlamydomonas has yielded a great deal of molecular genetic information on chloroplast gene expression (Rochaix, 1992). Both nuclear and chloroplast transformation techniques are well established (Boynton *et al.*, 1988; Debuchy *et al.*, 1989; Kindle *et al.*, 1989; Kindle, 1990), and for chloroplasts, gene disruption and co-transformation have allowed a wide variety of mutations to be introduced into the chloroplast genome (Goldschmidt-Clermont, 1991; Kindle *et al.*, 1991; Newman *et al.*, 1991). To begin studying the process of translation initiation in *Chlamydomonas* chloroplasts, we have introduced mutations into the initiation codon of the chloroplast *petD* gene, which encodes subunit IV of the cytochrome b_6/f complex (Lemaire *et al.*, 1986; Fong and Surzycki, 1992), an essential component of the photosynthetic electron transport chain. These mutations alter the initiation codon from AUG to either AUC or AUU. We report here that these mutations cause a temperature-sensitive photosynthetic growth defect, probably due to inefficient translation of *petD* mRNA. The analysis of such mutants, and the identification of suppressors that overcome the defect caused by the initiation codon mutation, are promising approaches to identifying components involved in the chloroplast translation initiation process.

Results

Creation of mutant Chlamydomonas strains using chloroplast transformation

Several *Chlamydomonas* strains carrying mutations in the chloroplast *petD* gene were created by particle bombardment, using a co-transformation strategy (Kindle *et al.*, 1991). A map of this region is shown in Figure 1. The *petA* gene, which encodes cytochrome f, is located upstream of *petD*, and a $tRNA^{Arg}$ gene is located immediately downstream (Büschlen *et al.*, 1991; Yu and Spreitzer, 1991). Strains iniD1 and iniD2, which carry AUG→AUC and AUG→AUU mutations in the initiation codon of the *petD* gene, respectively, were created by co-transformation of the wild-type *Chlamydomonas* strain P17 with plasmids containing these mutations, together with mutant 16S rDNA which confers spectinomycin resistance (see Materials and methods). After bombardment, the cells were first selected for spectinomycin resistance, and then screened for the presence of the initiation codon mutations in the *petD* gene, using the *Dde*I and *Ssp*I sites created by the AUC and AUU mutations, respectively (see Figure 1).

As shown in Figure 2, a 570 bp fragment of the *petD* locus, which spans the initiation codon of *petD*, could be amplified from total DNA by PCR, using primers WS6 and PD344. The PCR products were digested with either *Dde*I or *Ssp*I. While the PCR product from the wild-type recipient strain remained undigested, those from iniD1 and iniD2 transformants yielded two fragments of 250 bp and 320 bp (Figure 2B and C). Although most transformants were originally heteroplasmic, their heteroplasmicity was resolved following two rounds of single colony isolation. To establish that the *ini* strains were homoplasmic, we carried out DNA filter hybridizations to detect any undigested PCR fragment after the restriction enzyme digestion. Since the uncut PCR fragment either represents incomplete digestion or heteroplasmicity, we mixed PCR products from wild-type and mutant plasmids in ratios such as 1:40, 1:80 and 1:160, and digested them with same restriction enzymes under identical conditions. Since the chloroplast DNA copy number is at most 80 (Harris, 1988), one wild-type copy among other mutant copies should be represented by the 1:80 mixture. Since the amount of undigested PCR fragment from the *ini* mutants was equivalent to or less than the amount of undigested wild-type PCR product in the 1:160 mixture (data not shown), we concluded that the *ini* strains were homoplasmic. These homoplasmic strains were used for all further analyses.

We also created a mutant strain called ΔpetD, in which most of the *petD* coding region and part of its 5′ untranslated region are deleted (Figure 1). This strain was obtained by co-transformation of a wild-type strain with the deleted plasmid and the spectinomycin resistance marker. Spectinomycin-resistant colonies were screened by amplifying the *petD* locus with primers WS4 and WS7 (Figure 2A). Homoplasmic ΔpetD transformants gave rise to a 480 bp product, in contrast to a 830 bp product from wild-type cells (Figure 2D). The four independent ΔpetD transformants shown in Figure 2D were obtained after two

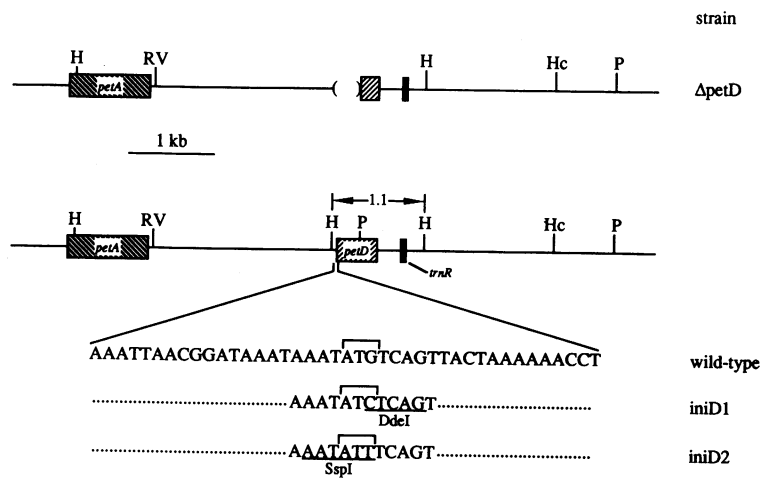

Fig. 1. Map of the *petA*−*petD* region of the *Chlamydomonas* chloroplast genome. The ΔpetD strain (top) carries a deletion of a 350 bp *Hin*dIII−*Pst*I fragment. The initiation codon changes in the iniD1 and iniD2 strains are shown, along with the restriction endonuclease sites that these changes create (bottom). Restriction sites are H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; RV, *Eco*RV.

rounds of single colony isolation, and are homoplasmic for the *petD* deletion.

Because iniD1, iniD2 and ΔpetD were created by co-transformation, these strains carry mutations both in chloroplast 16S rDNA and in *petD*. To ensure that the phenotypes of the mutant strains resulted from the mutations in the *petD* gene and not from the 16S rDNA mutation, we created a strain carrying only the 16S rDNA mutation, which

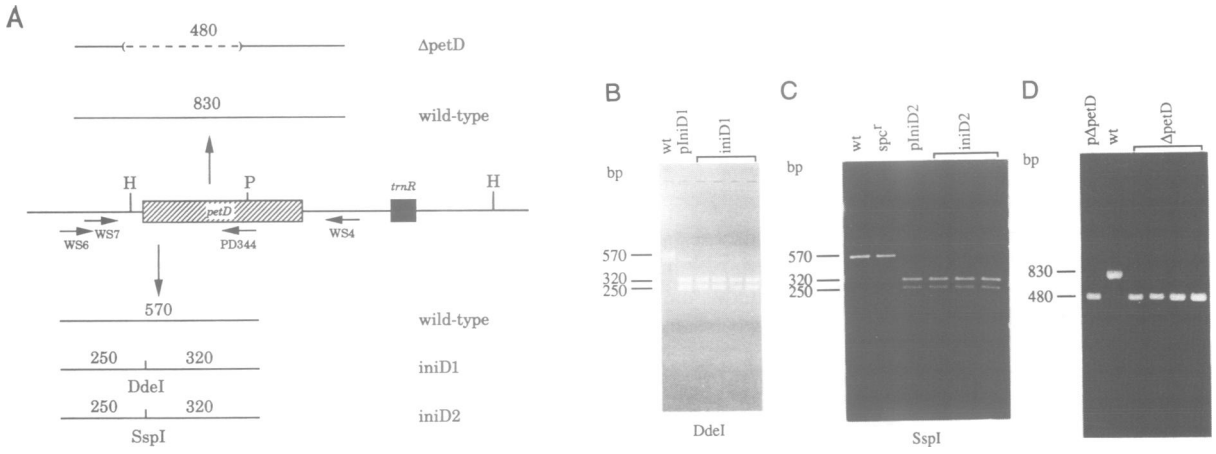

Fig. 2. Creation of ΔpetD, iniD1 and iniD2 strains by co-transformation. **(A)** Screening strategies to identify transformants carrying mutations. For ΔpetD transformants, DNA was amplified using primers WS4 and WS7, and products of 830 bp and 480 bp were produced from wild-type and mutant strains, respectively. For iniD1 and iniD2 transformants, DNA was amplified using primers WS6 and PD344. The PCR products from wild-type and mutant cells are the same size, but the mutant PCR products carry new restriction sites. **(B)** PCR amplification using primers WS6 and PD344, and DNA of the wild-type recipient strain (wt), the transforming plasmid pIniD1, and four independent transformants (iniD1), followed by digestion with *Dde*I. **(C)** PCR amplification using primers WS6 and PD344, and DNA of the wild-type recipient strain (wt), the strain that was transformed with spc^r 16S DNA alone (spc^r), the transforming plasmid pIniD2, and three independent co-transformants (iniD2), followed by digestion with *Ssp*I. **(D)** Amplification of the *petD* region using primers WS4 and WS7, and DNA of the transforming plasmid pΔpetD, the wild-type recipient strain (wt), and four independent transformants (ΔpetD).

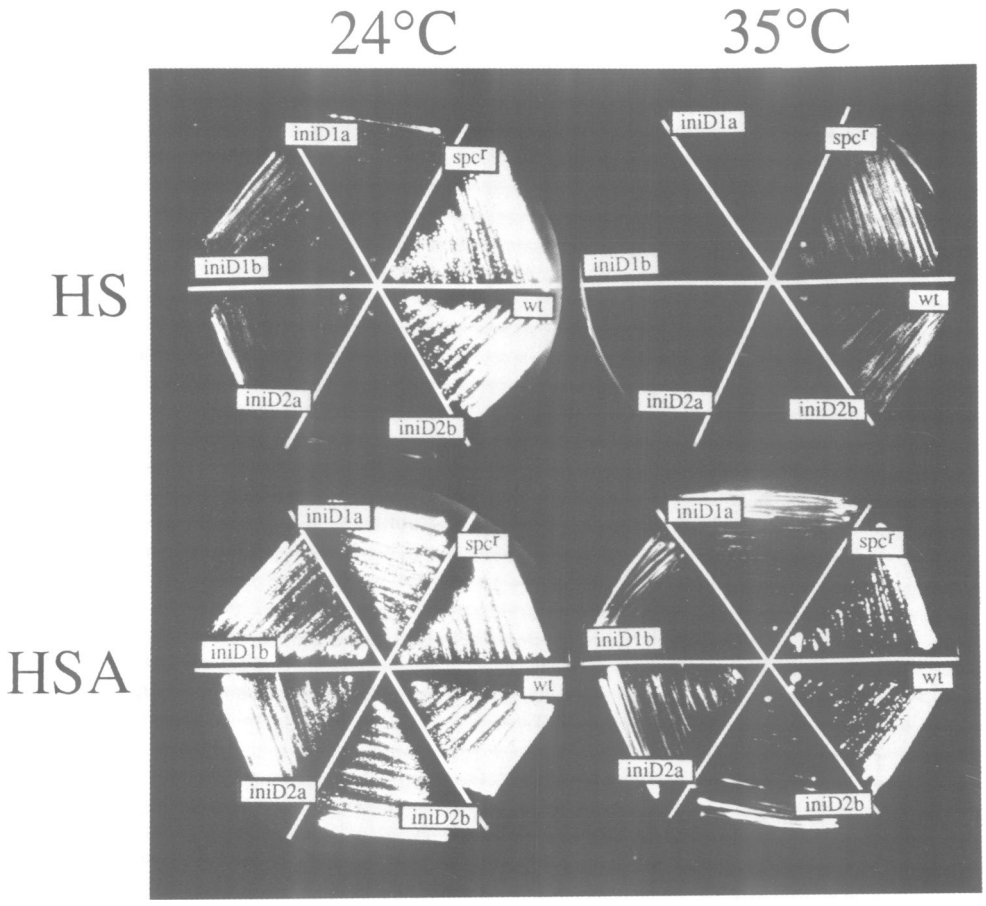

Fig. 3. Growth of iniD1, iniD2 and control strains. Wild-type (wt), spectinomycin-resistant (spc^r), iniD1 and iniD2 cells were streaked onto either HS or HSA plates and incubated at 24°C under medium light with a 16 h light, 8 h dark cycle, or at 35°C for 10 days under continuous medium light. The photograph was printed as a negative to make the colonies more visible.

will be referred to as spc^r. This strain was used as a control in all experiments and showed that the spc^r mutation has no effect on *petD* expression.

Growth of initiation codon mutants

The iniD1, iniD2 and control strains were first tested for their ability to grow phototrophically, by plating them on HS medium and incubating at 24°C under moderate light with a 16 h light, 8 h dark cycle. On HS plates, which lack acetate and do not support heterotrophic growth, the growth of spc^r was indistinguishable from the wild-type strain. ΔpetD was not able to grow phototrophically (data not shown), which was as expected since the cytochrome b_6/f complex is essential for photosynthesis. In contrast, iniD1 and iniD2 were able to grow phototrophically under these conditions (Figure 3). However, the growth rate was reduced compared with that of the wild-type and spc^r strains. To confirm this result independently, we transformed the ΔpetD strain with plasmids pIniD1 and pIniD2 and selected phototrophic transformants on HS plates. Numerous transformants were obtained with both plasmids, indicating that strains carrying mutations of the *petD* initiation codon to AUU or AUC do indeed support phototrophic growth at 24°C under moderate light conditions.

The reduced growth rate of the iniD1 and iniD2 strains reflected reduced photosynthetic capacity, because their growth rates were indistinguishable from those of the wild-type and spc^r strains on plates containing acetate (HSA) at 24°C, conditions under which photosynthesis is dispensable (Figure 3). The doubling time for wild-type, spc^r and iniD2 strains was determined in liquid HS medium at 24°C under moderate, continuous light with an ambient CO_2 concentration of 5%. Under these conditions, the doubling time of the wild-type and spc^r strains was 20 h, whereas the doubling time of the iniD2 strain was 30 h.

The growth characteristics of the iniD1 and iniD2 strains were tested under different conditions of light intensity and temperature: at 14°C with medium light, at 24°C with strong or medium light, and at 35°C with medium light. All strains were able to grow on HSA plates under all of the above conditions. However, iniD1 and iniD2 were more light sensitive than the wild-type and spc^r strains, as evidenced by progressive bleaching of the cells. iniD1 and iniD2 cells initially grew on HSA plates, but later stopped dividing, and became photobleached in 1 week at 35°C with medium light or at 24°C with strong light, or in 2–3 weeks at 24 or 14°C with medium light. Under all these conditions, the wild-type and the spc^r strains remained green for at least 6 weeks, when the plates were discarded. When grown on HS plates, the mutants exhibited abnormal phenotypes, which were more pronounced at higher temperatures or light intensities. At 24°C under continuous, medium-intensity light, the *ini* mutant strains grew at a much reduced rate and became bleached within 10 days after inoculation. At 24°C under continuous, high-intensity light, minimal growth was observed, and cells were bleached within a few days after inoculation. At 35°C under continuous, moderate light, the mutants failed to grow (Figure 3). We conclude that substitution of either AUU or AUC for the *petD* initiation codon results in temperature and light sensitivity for photosynthetic growth.

Subunit IV accumulation

The fact that the iniD1 and iniD2 strains were able to grow phototrophically at 24°C under medium light intensity indicated that subunit IV accumulated in these strains, even though the *petD* initiation codon had been mutated. We determined the steady-state levels of subunit IV and other proteins in wild-type and mutant strains by Western blotting. The strains were grown at 24°C in liquid HSA medium under dim light, a condition in which the cells do not require photosynthesis for growth. Figure 4A shows that subunit IV accumulated to ~10–20% of wild-type levels in the iniD1 and iniD2 strains, consistent with their weak phototrophic phenotype in the absence of acetate. Similar results were obtained with cells grown in HS medium (data not shown). We also determined the amount of cytochrome f, another

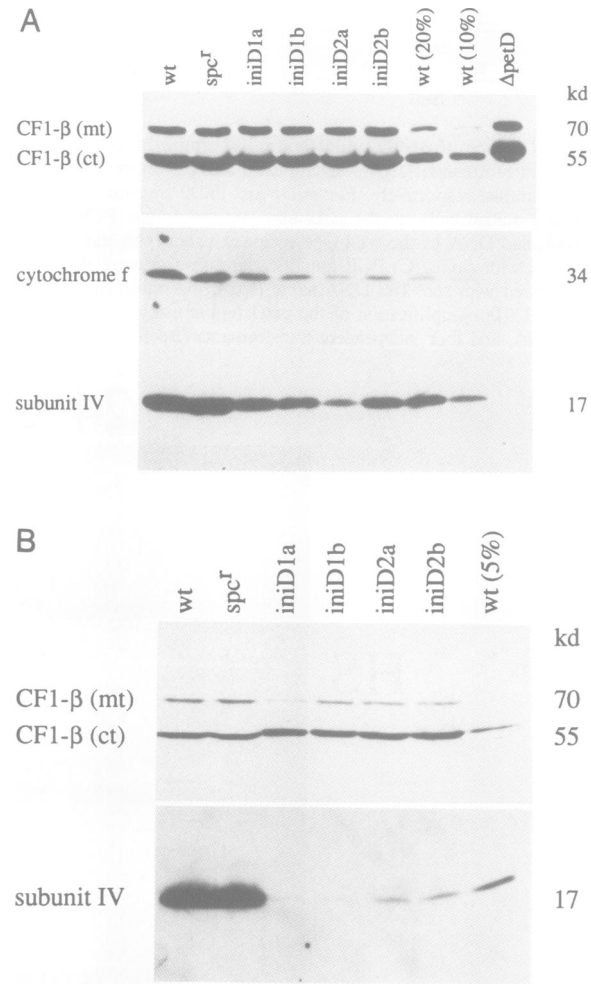

Fig. 4. Western blot analyses for the wild-type (wt), spectinomycin resistant (spc^r), iniD1, iniD2 and ΔpetD strains grown in HSA medium at 24°C (**A**), or grown on HSA plates at 35°C (**B**). Five-fold, 10-fold and 20-fold dilutions of wild-type proteins from cells grown under the same conditions as the mutants (wt 20%, wt 10%, wt 5%) were used to quantify subunit IV levels. The CF1-β antibody was used at a 1:10 000 dilution and purified subunit IV antibody at a 1:2000 dilution. iniD1a and iniD1b are two independent transformants that contain the AUC mutation, and iniD2a and iniD2b are two independent transformants that contain the AUU mutation. CF1-β (mt) and CF1-β (ct) represent the β-subunit of the mitochondrial and the chloroplast ATPases, respectively, which were used to normalize the amount of proteins in each lane.

component of the cytochrome b_6/f complex. In the ΔpetD strain, cytochrome f was undetectable. In the iniD1 and iniD2 strains, cytochrome f accumulated to ~10–20% of wild-type levels. The fact that cytochrome f accumulation parallels subunit IV accumulation indicates that the accumulation of these two components of the cytochrome b_6/f complex is coordinately regulated, as previously noted (Lemaire *et al.*, 1986).

The level of subunit IV accumulation in the iniD1 and iniD2 strains indicates that 10% of the wild-type amount of subunit IV is sufficient to support phototrophic growth at 24°C. Since the iniD1 and iniD2 strains did not grow phototrophically at 35°C, subunit IV accumulation must be too low at 35°C to support phototrophic growth. We therefore measured the amount of subunit IV in wild-type and mutant cells grown on HSA plates at 35°C. Figure 4B shows that in mutant cells, subunit IV accumulates to <5% of the wild-type amount. At 35°C, subunit IV accumulation in iniD1 (AUG→AUC) cells is more severely affected than in iniD2 (AUG→AUU) cells. Under these same conditions, wild-type cells accumulate 20% of the amount of subunit IV accumulated by wild-type cells grown at 24°C in HSA liquid culture, normalized relative to CF_1-β (the β-subunit of the ATPase; data not shown). Therefore, at 35°C, iniD1 and iniD2 accumulate ~1% of the subunit IV found in wild-type cells at 24°C. We conclude that the temperature sensitivity of photosynthetic growth is due to the lack of subunit IV accumulation at the restrictive temperature.

petD mRNA accumulation

To establish the molecular mechanism for reduced subunit IV accumulation in the iniD1 and iniD2 mutants, we first determined the accumulation of *petD* mRNA. Total cellular RNA was isolated from log phase cultures of strains grown in HSA medium, and *atpB* and *petD* mRNAs were detected by filter hybridizations using gene-specific probes. When *atpB* mRNA was used as a control to normalize the amount of RNA loaded in each lane, we found that the *petD* transcript accumulated to ~50% of wild-type levels in the iniD1 and iniD2 strains, as shown in Figure 5.

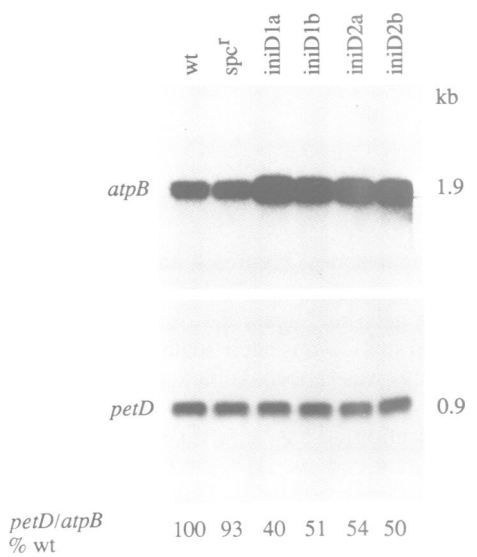

Fig. 5. RNA filter hybridization analysis of *petD* and *atpB* transcript levels. Total cellular RNA was separated in a 1.2% agarose–formaldehyde gel, transferred to nylon filters and hybridized with the WS6/PD344 PCR product, which is specific for the *petD* transcript, or with a probe specific for the *atpB* transcript. The amount of RNA loaded in the *ini* lanes was increased to equalize the *petD* mRNA signal. The relative level of *petD* mRNA is indicated below the gel for each strain.

Subunit IV and cytochrome f synthesis

Since the reduction in *petD* mRNA accumulation in the iniD1 and iniD2 strains could not fully account for the reduction in subunit IV accumulation, we investigated the rate of synthesis of subunit IV by pulse labeling thylakoid membrane proteins *in vivo*. We labeled cells from wild-type, spc^r, iniD1, iniD2 and ΔpetD strains with [^{35}S]Na_2SO_4 for 5 min to determine subunit IV synthetic rates, or for 45 min to increase the total incorporation of label and to examine short term accumulation of the cytochrome b_6/f subunits.

When cells were labeled for 5 min, the overall labeling patterns in the wild-type and spc^r strains were indistinguishable, indicating that the spc^r mutation does not affect the rate of synthesis of the proteins investigated here (Figure 6A). Under these conditions, subunit IV synthesis was detected at similar levels in the wild-type and spc^r strains, but was undetectable in the ΔpetD, iniD1 and iniD2 strains. If wild-type and *ini* mRNAs were equally translatable, 50% of the wild-type synthesis rate would be expected for the *ini* strains, corresponding to the relative mRNA accumulation, and this would be detectable in our gel system (unpublished results). When cells were labeled for 45 min, subunit IV was strongly labeled in the wild-type and spc^r strains (Figure 6B). When the amount of subunit IV labeling was quantified relative to each of three protein bands unrelated to the b_6/f complex, iniD1 and iniD2 had 10–20% of the wild-type level, consistent with the steady-state subunit IV levels (Table I). We conclude that the iniD1 and iniD2 mutations reduce the translational efficiency of *petD* mRNA.

The labeled protein bands corresponding to cytochrome f were identified by Western blotting. After 5 min of labeling (Figure 6A and Table I), cytochrome f synthesis in the iniD1 and iniD2 mutants was close to the level in wild-type strains. This indicates that the synthetic rate of cytochrome f in the iniD1 and iniD2 mutants is largely unaffected, and the reduced steady-state cytochrome f level determined by Western blotting is probably due to instability of unassembled cytochrome f in the mutants. After 45 min of labeling, the amount of labeled cytochrome f was 17% of the wild-type level in the ΔpetD strain, and 50% of the wild-type level in the iniD1 and iniD2 strains. This indicates that although cytochrome f is unstable in the mutant strains, the steady-state concentration has not been achieved after 45 min.

Discussion

Translation initiation in Chlamydomonas chloroplasts

In order to study the translation initiation process in *Chlamydomonas* chloroplasts, we introduced initiation codon mutations into the chloroplast *petD* gene by transformation. Strains carrying these mutations accumulate 50% of *petD* mRNA and 10–20% of subunit IV, relative to the wild-

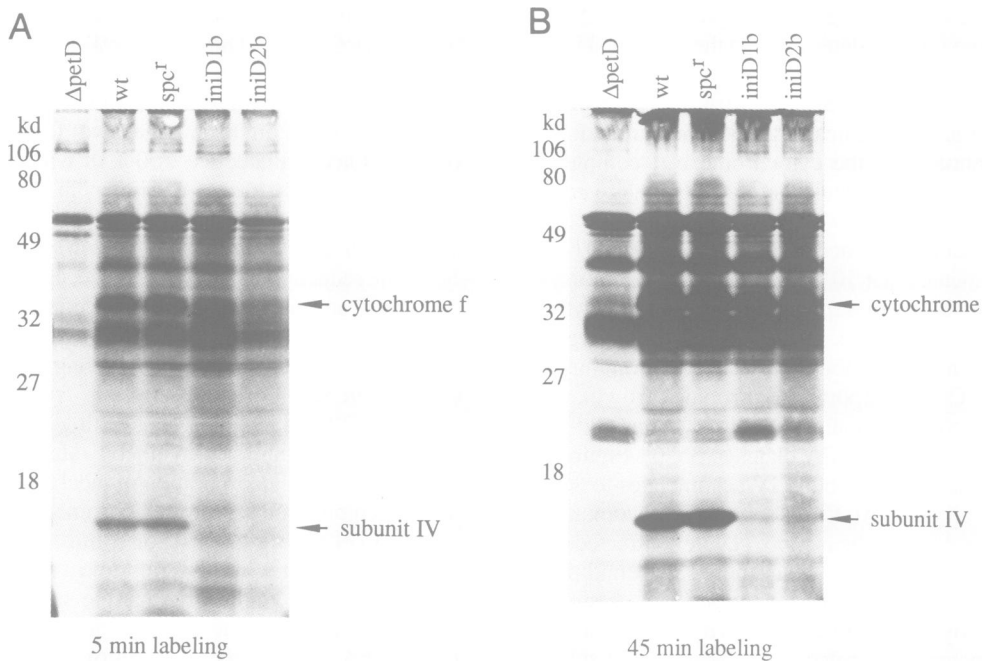

Fig. 6. Pulse-labeling of thylakoid membrane proteins of iniD1, iniD2 and control strains. Labeling and electrophoresis were carried out as described in Materials and methods. **(A)** shows results from a 5 min labeling and **(B)** shows results from a 45 min labeling. The identities of the bands labeled as cytochrome f and subunit IV were inferred by their apparent molecular masses of 32 and 17 kDa, respectively, and confirmed by their recognition by antibodies raised against the two proteins (data not shown). Subunit IV was absent in the ΔpetD strain. The quantitation of subunit IV and cytochrome f labeling is shown in Table I. The polypeptides that migrate just ahead of subunit IV in the 5 min labeling of iniD1 and iniD2 cells were not recognized by the subunit IV antibody. In addition, upon prolonged exposures of the gel, the same bands were seen in the ΔpetD lane. Thus, these are unlikely to represent truncated or degraded versions of subunit IV.

type strain. The reduced accumulation of subunit IV is due to a reduced synthetic rate of this protein from the mutant messages. This level of subunit IV accumulation is sufficient to support a reduced rate of phototrophic growth at 24°C. At 35°C, the strains fail to grow phototrophically. Since mutant strains growing heterotrophically at this temperature accumulated only trace levels of subunit IV, we conclude that at 35°C accumulation of this protein is insufficient to support phototrophic growth. The inability to grow at 35 versus 24°C could be a direct effect of elevated temperature on translation initiation at aberrant start codons, or it could be due to other effects of elevated temperature, for example on synthesis or accumulation of subunit IV or other proteins required for photosynthesis. Although this point could be addressed in principle by pulse-labeling of *ini* cells at 35°C under heterotrophic conditions, the low accumulation of subunit IV in these cells would make quantitative measurements of translation rates problematic.

Because the *ini* mutant strains were designed to utilize non-AUG translation initiation codons, we considered the possibility that an aberrant translation start site might be used. Examination of the DNA sequences surrounding the normal initiation codon revealed an in-frame UAA (stop) three codons upstream. This almost certainly rules out the possibility that the iniD1 and iniD2 mutants generate subunit IV from an upstream AUG codon. The first downstream, in-frame AUG is 21 codons downstream, which could possibly serve as the initiation codon in the iniD1 and iniD2 mutants. If this were the case, subunit IV would be truncated by 2 kDa, producing a 15 kDa protein that should be easily distinguishable from the 17 kDa wild-type protein in SDS−polyacrylamide gels. However, the migration of subunit IV protein produced in wild-type and mutant strains

Table I. Quantitation of subunit IV and cytochrome f labeling in the pulse-labeling experiment (Figure 6)

	% wt cytochrome f		% wt subunit IV
	5 min labeling	45 min labeling	45 min labeling
wt	100	100	100
spc^r	101	103	109
iniD1	84	49	11
iniD2	86	52	18

is indistinguishable (Figures 4 and 6). We also compared subunit IV migration in 12−18% gradient polyacrylamide, 8 M urea gels, and again the proteins from wild-type and mutant strains co-migrated (data not shown). N-terminal sequencing was not possible, because *Chlamydomonas* subunit IV is blocked (F.-A.Wollman, personal communication). Thus, it is very likely that the iniD1 and iniD2 mutant codons, AUC and AUU, serve as initiation codons that are less efficient than AUG. We cannot exclude the possibility that other non-AUG codons near the normal initiation codon serve as the initiation codon in the iniD1 and iniD2 mutants, and that the subunit IV produced in the mutants differs from the wild-type protein by one or a few amino acids, and appears to co-migrate with the wild-type protein in SDS gels.

The ability of the chloroplast translational apparatus to utilize a non-AUG initiation codon is not overly surprising, given its prokaryotic nature. In *E.coli*, although AUG (90%) and GUG (9%) account for most initiation codons (Hershey, 1987), use of AUU is known for *infC* mRNA (Butler *et al.*, 1987). AUC does not occur naturally as an initiation codon,

but it was tested for its ability to initiate translation in *E.coli* using genes for phage HB-3 murein hydrolase and β-galactosidase as reporters (Romero and Garcia, 1991). It was found that the relative translation efficiency for AUC-containing mRNA was 5% of that for AUG-containing mRNA. In chloroplasts, GUG is found in several cases; for example it is used in *Chlamydomonas psbC* mRNA (Rochaix *et al.*, 1989). ACG is also found in several chloroplast genes at the initiation codon position; however, this codon is altered to AUG by RNA editing (Hoch *et al.*, 1991; Kudla *et al.*, 1992). In yeast mitochondria, the *COX3* AUG initiation codon could be altered to AUA with a 20% retention of translational competence (Folley and Fox, 1991).

The iniD1 and iniD2 strains are phototrophic at 24°C, although they are substantially reduced in the synthesis and accumulation of subunit IV with respect to wild-type strains. Similarly, the expression of other photosynthetic components can be reduced without rendering the cells non-photosynthetic. For example, a photosynthetic revertant of the FUD44 strain which lacks photosynthetic oxygen-evolving activity displays only 30% of oxygen-evolving activity of wild-type cells (Mayfield *et al.*, 1987). F34 suI, which is a chloroplast suppressor of the nuclear mutant F34 that is unable to translate *psbC* mRNA, synthesizes and accumulates between 20 and 25% of wild-type levels of the PSII chlorophyll apoprotein CP43 and is able to photosynthesize (Rochaix *et al.*, 1989). These data imply that in wild-type strains, many components of the photosynthetic apparatus are present in excess of the amounts needed to support photosynthetic growth.

Initiation codon selection in *Chlamydomonas* chloroplasts is probably based on sequence context beyond the AUG codon and the immediately surrounding nucleotides, and these other determinants may facilitate correct initiation in the *ini* mutants. One such element might be a ribosome binding site, which has been proposed based on sequence analysis (Bonham-Smith and Bourque, 1989) but has not been functionally identified in chloroplasts. A second element could be upstream sequences that serve as recognition sites for translational activators or repressors. Genetic evidence for such an element has been obtained for the *Chlamydomonas psbC* mRNA (Rochaix *et al.*, 1989), and a candidate protein factor has been purified based on its affinity for the *Chlamydomonas psbA* 5′ UTR (Danon and Mayfield, 1991). In addition, deletion mutagenesis of the *petD* 5′ UTR (Sakamoto *et al.*, 1993; our unpublished results) and the characterization of several nuclear mutants that affect translation of chloroplast ATPase, photosystem and electron transport protein mRNAs (Rochaix, 1992) argue that *trans*-acting factors may have a general role in *Chlamydomonas* chloroplast translation, and may outweigh the need for a correctly positioned AUG codon.

Differential petD transcript accumulation in ini mutants

The observation that *petD* mRNA accumulates to 50% of the wild-type level in the iniD1 and iniD2 strains is intriguing. Since the mutations in iniD1 and iniD2 are single base changes far from the promoter region, it is unlikely that the transcription rate of *petD* is altered in these mutants. It is also unlikely that these mutations would alter the structures of the mutant messages and therefore their stabilities. Protein pulse-labeling experiments show that the mutants have reduced subunit IV synthetic rates, presumably because of reduced translation initiation. We propose that the direct effect of the *ini* mutations is on translation initiation, and that inefficient translation initiation has a secondary effect on transcript stability. Similarly, in strains carrying deletions in the 5′ UTR of *petD* mRNA, mRNA accumulation is reduced only when the deletion reduces or abolishes translation of the message (Sakamoto *et al.*, 1993; W.Sakamoto, K.Kindle and D.B.Stern, unpublished results). The simplest explanation for this observation is that ribosomes physically protect the mRNAs; therefore, if a mutant *petD* mRNA cannot be efficiently translated, the number of ribosomes on the mRNA is reduced and nucleases have more access to the mRNA. Both increases and decreases in mRNA stability have been related to translatability or polysome association for several other chloroplast mRNAs (Klaff and Gruissem, 1991; Drapier *et al.*, 1992; Barkan, 1993), and also for cytosolic animal cell mRNAs (Atwater *et al.*, 1990). The mechanisms that link chloroplast mRNA stability and translation remain poorly understood (Rochaix, 1992).

Coordinate regulation of components in cytochrome b_6/f complex

The cytochrome b_6/f complex consists of five (Lemaire *et al.*, 1986; Haley and Bogorad, 1989; Fong and Surzycki, 1992) and possibly additional (Hurt and Hauska, 1982) subunits: the chloroplast-encoded cytochrome f, cytochrome b_6, and subunits IV and V, and the nuclear-encoded Rieske iron–sulfur protein. We have shown that in ΔpetD, iniD1 and iniD2 strains, in which subunit IV is either absent or reduced, the amount of cytochrome f is correlated with the amount of subunit IV. Preliminary results also show that the steady-state level of the nuclear-encoded Rieske iron–sulfur protein also correlates with the level of subunit IV (G.Parks and D.B.Stern, unpublished results). Pulse-labeling experiments showed that the reduced cytochrome f level in the mutants is not due to reduced synthesis, but rather to reduced stability in the mutants. Coordinate regulation at the post-translational level has been previously observed for the cytochrome b_6/f complex (Barkan *et al.*, 1986; Lemaire *et al.*, 1986; Bruce and Malkin, 1991) as well as for other photosynthetic complexes (Bennoun *et al.*, 1986; Jensen *et al.*, 1986; Girard-Bascou *et al.*, 1987; Mayfield *et al.*, 1987).

Materials and methods

Construction of petD transforming plasmids

pHH1.1 contains a 1.1 kb *Hin*dIII subfragment of *Bam*HI fragment 7 (Bam 7) from the *Chlamydomonas* chloroplast genome (Harris, 1988). It contains the *petD* coding region and a *trnR* gene.

Site-directed mutagenesis to create mutants in the initiation codon of *petD* was carried out on pHH1.1 according to Kunkel (1985). The sequences of the primers used for mutagenesis are: 5′-TTAGTAACTGA(G or A)ATA-TTTATTTATCCG-3′, (G, iniD1; A, iniD2) corresponds to nucleotides 375–349 with respect to the *petD* mRNA 5′ end (initiation codon underlined; Sakamoto *et al.*, 1993).

Following mutagenesis, upstream and downstream regions were cloned into this plasmid to obtain the larger plasmids pIniD1 and pIniD2, which contain the 5 kb *Eco*RV–*Hin*cII subfragment of the Bam 7 fragment (Figure 1). Plasmid pΔpetD contains the 5.7 kb *Eco*RV–*Pst*I fragment from the Bam 7 fragment, with a 350 bp deletion in *petD* gene (Figure 1). The deletion covers 42 bp in the 5′ untranslated region and 317 bp in the coding region, which corresponds to the fragment flanked by the *Hin*dIII site and the *petD*-internal *Pst*I site.

Chlamydomonas strains, growth conditions and chloroplast transformation

P17 (Stern *et al.*, 1991) is a wild-type *Chlamydomonas* strain derived from CC373 (ac-u-c-2-21; Shepard *et al.*, 1979) by bombardment with a wild-type *atpB* gene and was used to create mutant strains spc^r, iniD1 and iniD2 used in this study. spc^r was created by selecting a spc^r strain after bombardment with CrBH4.8 (Newman *et al.*, 1990) which carries a mutation in 16S rDNA.

Chlamydomonas strains were grown either in HS or HSA medium (Harris, 1988). Light conditions used were high (18–26 W/m^2), medium (5–8 W/m^2) and low (~1.3 W/m^2). Unless stated otherwise, cells were grown under continuous light.

Chloroplast co-transformation with CrBH4.8 (spc^r) was as described (Kindle *et al.*, 1991), except that a 2:1 ratio of *petD* DNA to CrBH4.8 DNA was used, and *petD* DNA was linearized to increase the efficiency of transformation. Since co-transformants were expected to be deficient in subunit IV of the cytochrome b_6/f complex, plates were loosely wrapped in aluminum foil to provide dim light after bombardment.

Chlamydomonas DNA minipreps and PCR

Approximately 10^4 cells were scraped from a plate and resuspended in 100 μl of proteinase K buffer (10 mM Tris–Cl, pH 8, 10 mM EDTA, 10 mM NaCl), SDS was added to a final concentration of 2.5% and proteinase K was added to a final concentration of 200 μg/ml. The samples were incubated at 55°C for 1 h, extracted with phenol/chloroform, and the DNA was precipitated with ethanol. The DNA was dissolved in 15 μl of H_2O, and 5 μl were used for PCR.

The match between PCR primers used for screening transformants and the *petD* sequence are illustrated in Figure 2; each contained additional 5′ nucleotides that added restriction sites. The sequences were as follows: WS4, 5′-TGGAGCTCGTCTAGAGGAATCTCCTATTTTGTAGGATG-3′; WS7, 5′-CCAGATCTGAACAAATGCCAAAATCTACT-3′; WS6, 5′-AAAGATCTTTTCGGAACGGCTAAACC-3′; and PD344, 5′-CCTGCAGGTACTGCTGC-3′. The primers used for amplifying the *petD* coding region for fusion protein production were WS4 and FP001. The additional 5′ sequences in WS4 create *Sac*I and *Xba*I sites. FP001, 5′-AAAGATATCATGTCAGTTACTAAAAAACC-3′, corresponds to nucleotides 360–380 of the *petD* sequence with nine additional nucleotides that create an *Eco*RV site. The PCR reactions were carried out using 1.5 mM $MgCl_2$, 40 μM dNTPs, 100 μg of each primer, and 2 U of Promega *Taq* polymerase for each reaction. 30 cycles of amplification were carried out at an annealing temperature of 55°C and a reaction temperature of 72°C.

Chlamydomonas total protein preparation and Western blotting

Chlamydomonas strains were grown in HSA medium until the cultures reached late log phase or were harvested from HSA plates. 10^7 cells were harvested by centrifugation at 2000 r.p.m. in a bench-top centrifuge and either quick-frozen and stored at −80°C, or resuspended in 100 μl of protein sample buffer (50 mM Tris pH 6.8, 5% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and boiled for 5 min. After pelleting insoluble material, 10% of the supernatant was loaded into a 15% SDS–polyacrylamide gel, which was electrophoresed at 25 mA until the chlorophyll reached the bottom of the gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane by electroblotting. Blocking and reaction with antibodies were carried out as described (Stern *et al.*, 1991). Antibodies raised against spinach CF1-β were obtained from R.McCarty (Johns Hopkins University), and antibodies directed against *Chlamydomonas* subunit IV were generated as described below. HRP-linked goat anti-rabbit antibodies were purchased from Promega and used at a 1:2000 dilution. The Amersham ECL chemiluminescent detection technique was used to visualize immunoreactive bands.

To raise antibodies against *Chlamydomonas* subunit IV, the *petD* coding region was amplified using primers FP001 and WS4 and an *Eco*RV–*Xba*I fragment was cloned into the fusion protein vector pGEX-3X, which generates glutathione-*S*-transferase fusion proteins in *E.coli* (Smith and Johnson, 1988). The glutathione-*S*-transferase–subunit IV fusion protein was found to be insoluble, and could not be purified using glutathione affinity chromatography. It was therefore gel-purified as follows. 10 ml of an overnight culture of *E.coli* was inoculated into 100 ml M9ZB (10 g Bacto tryptone, 5 g of yeast extract and 5 g NaCl per liter) and grown at 37°C for 1.5 h, IPTG was added to 0.1 mM and cell growth continued for 2 h. The cells were harvested, resuspended in 6 ml of SDS sample buffer, boiled for 10 min, electrophoresed in preparative 12% SDS–polyacrylamide gels. The fusion protein was excised from the gel, electroeluted and quantified by SDS–PAGE using BSA standards (Promega). 100 μg of the fusion protein was initially injected into a rabbit followed by two boosters of 50 μg each. The crude serum identified subunit IV along with five to six unknown proteins in *Chlamydomonas* total protein extracts. A maltose binding protein–subunit IV fusion protein construct was made by cloning the FP00l/WS4 PCR product from HH1.1 into the *Stu*I and *Xba*I sites of the pMAL-p vector (Maina *et al.*, 1988). Total proteins from uninduced and induced cells were loaded into preparative SDS–polyacrylamide gels and transferred to nitrocellulose membranes. A small strip of the membrane was used to detect the induced fusion protein band by Western blotting using crude serum against glutathione-*S*-transferase–subunit IV fusion protein. The remainder of the membrane was incubated with crude GST-*petD* antiserum at a 1:15 dilution overnight at 4°C. The part of the membrane corresponding to the induced maltose binding protein–subunit IV fusion protein was excised and the antibodies were eluted in 1 ml of 100 mM glycine (pH 2.5). The eluant was neutralized with 0.1 vol of 1 M Tris (pH 8), concentrated, and subunit IV specificity was confirmed using blots of total *Chlamydomonas* proteins from wild-type and Δ*petD* strains.

RNA isolation and RNA filter hybridizations

Cells were grown to mid-log phase in HSA medium, RNA was isolated and filter hybridizations were carried out as described (Stern *et al.*, 1991). *atpB* mRNA was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amount of *petD* mRNA was determined by scanning the X-ray film into a TIFF file and quantifying the densities of bands using the public domain program Image (v. 1.33g).

In vivo pulse-labeling and crude thylakoid membrane preparation

1 ml (10^6) *Chlamydomonas* cells grown in liquid HSA were diluted into 100 ml of low sulfur HSA medium (in which $MgSO_4$ replaced $MgCl_2$) and grown to a density of 2 × 10^6 cells/ml. 50 ml of cells were harvested by low speed centrifugation (2000 *g*), washed and the cell pellets were resuspended in 1 ml of HSA lacking sulfur, prepared by leaving out the trace elements from low sulfur HSA medium. The cells were incubated in this medium for 45 min while stirring on a magnetic plate. Cycloheximide was then added to a final concentration of 8 μg/ml and 10 min later, 0.5 mCi of [^{35}S]Na_2SO_4 was added to each culture. After 5 or 45 min, the labeling reaction was stopped by the addition of 1 M Na_2SO_4 to a final concentration of 20 mM. Crude thylakoid membranes were prepared as described (Howe and Merchant, 1992) with the following modification. After the labeling reaction, the cells were washed with 1 ml of 50 mM Na-tricine (pH 8), 1 mM PMSF, 1 mM benzamidine, 5 mM caproic acid, 50 μg/ml aprotinin and 1 mM EDTA, and resuspended in 1 ml of the same buffer. Then the cells were broken using a French Press at 700 p.s.i., and the lysate was centrifuged at 30 000 *g* for 10 min at 4°C to pellet the membranes. The membrane pellets were resuspended in 30 μl of SDS sample buffer, boiled and loaded into a 15% SDS–polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes and exposed to the PhosphorImager to visualize and quantify the labeled proteins. The membrane was probed with antibodies against both subunit IV and cytochrome f to confirm their identities in the labeling gel. The membrane was exposed to X-ray film for 9 days for the 45 min labeling, and 42 days for the 5 min labeling.

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References

Atwater,J.A., Wisdom,R. and Verma,I.M. (1990) *Annu. Rev. Genet.*, **24**, 519–541.

Barkan,A. (1988) *EMBO J.*, **7**, 2637–2644.

Barkan,A. (1993) *The Plant Cell*, **5**, 389–402.

Barkan,A., Miles,D. and Taylor,W.C. (1986) *EMBO J.*, **5**, 1421–1428.

Büschlen,S., Choquet,Y., Kuras,R. and Wollman,F.-A. (1991) *FEBS Lett.*, **284**, 257–262.

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.

Bonham-Smith,P.C. and Bourque,D.P. (1989) *Nucleic Acids Res.*, **17**, 2057–2080.

Boni,I.V., Isaeva,D.M., Musychenko,M.L. and Tzareva,N.V. (1991) *Nucleic Acids Res.*, **19**, 155–162.
Boynton,J.E. *et al.* (1988) *Science*, **240**, 1534–1538.
Bruce,B.D. and Malkin,R. (1991) *The Plant Cell*, **3**, 203–212.
Butler,J.S., Springer,M. and Grunberg-Manago,M. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 4022–4025.
Danon,A. and Mayfield,S.P.Y. (1991) *EMBO J.*, **10**, 3993–4002.
Debuchy,R., Purton,S. and Rochaix,J.-D. (1989) *EMBO J.*, **8**, 2803–2809.
Drapier,D., Girard-Bascou,J. and Wollman,F.-A. (1992) *The Plant Cell*, **4**, 283–295.
Folley,L.S. and Fox,T.D. (1991) *Genetics*, **129**, 659–668.
Fong,S.E. and Surzycki,S.J. (1992) *Curr. Genet.*, **21**, 527–530.
Franzetti,B., Carol,P. and Mache,R. (1992) *J. Biol. Chem.*, **267**, 19075–19081.
Girard-Bascou,J., Choquet,Y., Schneider,M., Delosme,M. and Dron,M. (1987) *Curr. Genet.*, **12**, 489–496.
Gold,J.C. and Spremulli,L.L. (1985) *J. Biol. Chem.*, **260**, 14897–14900.
Gold,L., Pribnow,D., Schneider,T., Shinedling,S., Singer,B.S. and Stormo,G. (1981) *Annu. Rev. Microbiol.*, **35**, 365–403.
Goldschmidt-Clermont,M. (1991) *Nucleic Acids Res.*, **19**, 4083–4090.
Haley,J. and Bogorad,L. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 1534–1538.
Harris,E.H. (1988) *The Chlamydomonas Sourcebook*. Academic Press Inc., San Diego, CA.
Hershey,J.W.B. (1987) In Neidhardt,F.C. (ed.), *Escherichia coli and Salmonella typhimurium*. American Society for Microbiology, Washington, DC, Vol. 1, pp. 614–640.
Hoch,B., Maier,R.M., Appel,K., Igloi,G.L. and Koessel,H. (1991) *Nature*, **353**, 178–180.
Howe,G. and Merchant,S. (1992) *EMBO J.*, **11**, 2789–2801.
Hudson,G.S., Mason,J.G., Holton,T.A., Koller,B., Cox,G.B., Whitfeld,P.R. and Bottomley,W. (1987) *J. Mol. Biol.*, **196**, 283–298.
Hurt,E. and Hauska,G. (1982) *J. Bioenerg. Biomembr.*, **14**, 405–423.
Jensen,K.H., Herrin,D.L., Plumley,F.G. and Schmidt,G.W. (1986) *J. Cell Biol.*, **103**, 1315–1326.
Kindle,K.L. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 1228–1232.
Kindle,K.L., Richards,K.L. and Stern,D.B. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 1721–1725.
Kindle,K.L., Schnell,R.A., Fernandez,E. and Lefebvre,P.A. (1989) *J. Cell Biol.*, **109**, 2589–2602.
Klaff,P. and Gruissem,W. (1991) *Plant Cell*, **3**, 517–529.
Kraus,B.L. and Spremulli,L.L. (1986) *J. Biol. Chem.*, **261**, 4781–4784.
Kudla,J., Igloi,G.L., Metzlaff,M., Hagemann,R. and Koessel,H. (1992) *EMBO J.*, **11**, 1099–1103.
Kunkel,T.A. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 488–492.
Lemaire,C., Girard-Bascou,J., Wollman,F.-A. and Bennoun,P. (1986) *Biochim. Biophys. Acta*, **851**, 229–238.
Maina,C.V., Riggs,P.D., Grandea,A.G.,III, Slatko,B.E., Moran,L.S., Tagliamonte,J.A., McReynolds,L.A. and Guan,C.D. (1988) *Gene*, **74**, 365–374.
Mayfield,S.P., Bennoun,P. and Rochaix,J.-D. (1987) *EMBO J.*, **6**, 313–318.
Newman,S.M., Boynton,J.E., Gillham,N.W., Randolph-Anderson,B.L., Johnson,A.M. and Harris,E.H. (1990) *Genetics*, **126**, 875–888.
Newman,S.M., Gillham,N.W., Harris,E.H., Johnson,A.M. and Boynton,J.E. (1991) *Mol. Gen. Genet.*, **230**, 65–74.
Rochaix,J.-D. (1992) *Annu. Rev. Cell Biol.*, **8**, 1–28.
Rochaix,J.-D., Kuchka,M., Mayfield,S., Schirmer-Rahire,M., Girard-Bascou,J. and Bennoun,P. (1989) *EMBO J.*, **8**, 1013–1022.
Rock,C.D., Barkan,A. and Taylor,W.C. (1987) *Curr. Genet.*, **12**, 69–77.
Romero,A. and Garcia,P. (1991) *FEBS Lett.*, **84**, 325–330.
Ruf,M. and Koessel,H. (1988) *FEBS Lett.*, **240**, 41–44.
Sakamoto,W., Kindle,K.L. and Stern,D.B. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 497–501.
Shepard,H.S., Boynton,J.E. and Gillham,N.W. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 1353–1357.
Smith,D.B. and Johnson,K.S. (1988) *Gene*, **67**, 31–40.
Staub,J.M. and Maliga,P. (1993) *EMBO J.*, **12**, 601–605.
Stern,D.B., Radwanski,E.R. and Kindle,K.L. (1991) *Plant Cell*, **3**, 285–297.
Suguira,M. (1992) *Plant Mol. Biol.*, **19**, 149–168.
Westhoff,P. and Herrmann,R.G. (1988) *Eur. J. Biochem.*, **171**, 551–564.
Yu,W. and Spreitzer,R.J. (1991) *Nucleic Acids Res.*, **19**, 957.

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