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Transit Peptide Mutations That Impair in Vitro and in Vivo Chloroplast Protein Import Do Not Affect Accumulation of the γ-Subunit of Chloroplast ATPase[1]

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We have begun to take a genetic approach to study chloroplast protein import in *Chlamydomonas reinhardtii* by creating deletions in the transit peptide of the γ-subunit of chloroplast ATPase-coupling factor 1 (CF_1-γ, encoded by *AtpC*) and testing their effects in vivo by transforming the altered genes into an *atpC* mutant, and in vitro by importing mutant precursors into isolated *C. reinhardtii* chloroplasts. Deletions that removed 20 or 23 amino acid residues from the center of the transit peptide reduced in vitro import to an undetectable level but did not affect CF_1-γ accumulation in vivo. The CF_1-γ transit peptide does have an in vivo stroma-targeting function, since chimeric genes in which the stroma-targeting domain of the plastocyanin transit peptide was replaced by the *AtpC* transit peptide-coding region allowed plastocyanin to accumulate in vivo. To determine whether the transit peptide deletions were impaired in in vivo stroma targeting, mutant and wild-type *AtpC* transit peptide-coding regions were fused to the bacterial *ble* gene, which confers bleomycin resistance. Although 25% of the wild-type fusion protein was associated with chloroplasts, proteins with transit peptide deletions remained almost entirely cytosolic. These results suggest that even severely impaired in vivo chloroplast protein import probably does not limit the accumulation of CF_1-γ.

Most chloroplast proteins are encoded in the nucleus, synthesized in the cytosol as precursors, and imported into the chloroplast posttranslationally. The targeting information for translocation across the chloroplast envelope membranes resides in an N-terminal TP (for reviews, see Keegstra et al., 1989; de Boer and Weisbeek, 1991; Theg and Scott, 1993; Gray and Row, 1995; Schnell, 1995; Cline and Henry, 1996). Proteins destined for chloroplast compartments other than the stroma require additional targeting information. For inner envelope and integral thylakoid membrane proteins, the targeting signal appears to be in the mature protein. Precursors of thylakoid lumen proteins have a bipartite TP. The N-terminal part contains the stroma-targeting domain, which is removed by stromal processing to yield a translocation intermediate. The C-terminal part contains a hydrophobic region resembling bacterial signal sequences that directs translocation across the thylakoid membrane into the lumen using one of two targeting pathways (for reviews, see Keegstra, 1989; Robinson and Klösgen, 1994).

Although there is no primary amino acid sequence consensus among the stroma-targeting domains of TPs, they do share certain features. For example, they are enriched in basic and hydroxylated residues and lack acidic residues and Tyr. The stroma-targeting domains of vascular plant TPs have three domains: (a) an N-terminal part that is deficient in charged residues, Gly, and Pro; (b) a middle domain that is enriched in Ser, Thr, Lys, and Arg; and (c) a C-terminal portion that is predicted to form an amphiphilic β-strand (von Heijne et al., 1989). *Chlamydomonas reinhardtii* TPs are significantly shorter than those of vascular plants. Although their stroma-targeting domains are also predicted to have a tripartite domain structure, the uncharged N-terminal region is short, and the central region has a predicted α-helical nature more typical of a mitochondrial targeting signal. As in vascular plants, the C-terminal part is predicted to form an amphiphilic β-strand (Franzén et al., 1990).

Numerous in vitro studies have established that the TP is both necessary and sufficient for targeting chloroplast or foreign proteins to chloroplasts (for reviews, see Keegstra et al., 1989; de Boer and Weisbeek, 1991). In most cases, deletions throughout the TP have dramatic effects on in vitro import (Reiss et al., 1987; Archer and Keegstra, 1993; Bassham et al., 1994; Pilon et al., 1995; Lawrence and Kindle, 1997), suggesting that most parts of the TP contribute to the import process. Mutations in various parts of the stroma-targeting domain affect binding, translocation, and stromal processing to different degrees. However, the results have differed somewhat among various TPs; therefore, a detailed model for functional regions of the stroma-targeting region has not emerged. The N terminus appears to be involved in binding to and translocation across the envelope, whereas the C terminus plays a stronger role in defining the site and efficiency of stromal processing (Pilon et al., 1995; Cline and Henry, 1996).

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Abbreviations: BRP, bleomycin-resistance protein; CF_1-β and CF_1-γ, β- and γ-subunits of chloroplast ATPase-coupling factor 1; PC, plastocyanin; SSU, small subunit of Rubisco; TP, transit peptide.

It is generally assumed that in vitro import assays accurately reflect processes that occur in vivo, although this assumption has not been rigorously tested. In fact, different results were obtained in vivo and in vitro with two constructs in which the SSU TP-coding region was fused to that of neomycin phosphotransferase (*Npt*II) with or without 23 amino acid residues from the N terminus of a mature SSU (Kuntz et al., 1986; Wasmann et al., 1986). Similarly, a fusion of the PC TP to β-lactamase resulted in a chimeric precursor that was correctly localized to the lumen in vivo but not in vitro (de Boer et al., 1991). Fusion proteins that included the TP and 5 or 23 N-terminal amino acid residues of a chloroplast inner envelope protein were imported into both chloroplasts and mitochondria in vitro. However, in vivo the shorter fusion protein remained in the cytosol, and the longer one was localized in chloroplasts (Silva-Filho et al., 1997). The interpretations in these studies have been somewhat limited, because they involved chimeric proteins rather than native chloroplast proteins and were tested using different species for in vitro and in vivo analyses.

To investigate the mechanism of chloroplast protein import using the powerful in vivo and genetic approaches available for *C. reinhardtii*, we have made deletions throughout the TPs of two different chloroplast proteins: PC, a lumen-localized protein, and the γ-subunit of chloroplast ATPase, an extrinsic thylakoid membrane protein. The effects of these mutations have been examined in vivo, by transforming the mutant genes into *C. reinhardtii*, and in vitro, by importing mutant precursors into isolated *C. reinhardtii* chloroplasts. For PC, the in vivo and in vitro results agreed in most cases. However, deletions generally affected in vitro import more significantly than in vivo protein accumulation. An exception was a deletion that removed residues near the N terminus of the TP, which severely impaired in vivo protein accumulation, but affected in vitro import only moderately (Lawrence and Kindle, 1997).

We report here the disparate effects of TP mutations on in vivo accumulation and in vitro import of the γ-subunit of chloroplast ATPase. The chloroplast thylakoid ATPase uses the proton gradient generated by photosynthetic electron transport to synthesize ATP and therefore is required for photoautotrophic growth. Chloroplast ATPase has two discrete parts: CF_0, which is an integral thylakoid membrane protein complex composed of four polypeptides that carry out proton translocation, and CF_1, which has five polypeptide substituents with catalytic and regulatory activity. Three of the CF_1 subunits (α, β, and ε) are encoded by the chloroplast genome and two (γ and δ) are encoded in the nucleus (for reviews, see McCarty and Carmeli, 1982; Futai et al., 1989; Junge, 1989). CF_1-γ is an extrinsic, stroma-facing thylakoid membrane protein that is encoded by the nuclear *AtpC* gene. *C. reinhardtii* cDNA (Yu et al., 1988) and genomic (Smart and Selman, 1991b) clones have been isolated and sequenced. *C. reinhardtii* CF_1-γ is synthesized as a precursor and can be imported into pea (*Pisum sativum* L.) chloroplasts (Yu et al., 1988). A synthetic peptide identical to the 35-amino acid residue CF_1-γ TP competes with pea SSU for import into pea chloroplasts and is presumed to have a stroma-targeting function (Theg and Geske, 1992). Additional information for assembly of CF_1-γ into the ATPase complex presumably resides within the mature protein.

We have made a series of deletions in the TP-coding region of *AtpC* and tested their ability to complement the ATPase deficiency of an *atpC1* mutant (Smart and Selman, 1991a). CF_1-γ precursors containing the same deletions have been synthesized and tested in an in vitro *C. reinhardtii* chloroplast protein-import assay. As described below, the consequences of these mutations were very different in these two assays. By expressing chimeric genes in *C. reinhardtii* transformants, we demonstrate that the CF_1-γ TP has an in vivo chloroplast-targeting function, which is compromised by deletions identical to ones that have no discernible effect on CF_1-γ accumulation. We discuss the implications of these results and speculate that chloroplast protein import may not become the rate-limiting step for ATPase accumulation until it is impaired beyond even our most severe mutations.

MATERIALS AND METHODS

Chlamydomonas reinhardtii Strains

C. reinhardtii TI-54 *(nit1–305atpC1 mt⁻*; Smart and Selman, 1991a) was obtained from Bruce Selman (University of Wisconsin, Madison). It is also available from the Chlamydomonas Genetics Center at Duke University (Durham, NC) as CC-3022 and is referred to as such throughout this paper. Photosynthetic revertants of CC-3022 were selected at a rate of 10^{-7} by plating cells onto high-salt plates (Harris, 1989) and incubating them in the light for about 6 weeks. P17 is a wild type with respect to *AtpC* (Stern et al., 1991). AP6 *(arg7pcy1–1 mt⁺)* carries a mutation in the *pcy1* locus, which contains the structural *PetE* gene that encodes PC (Quinn et al., 1993; K.L. Kindle, unpublished data); it is available from the Chlamydomonas Genetics Center as CC-3371. Strain UG126 (CC-3396; *arg7nit1NIT2 mt-)* was obtained from E. Orr and U. Goodenough (Washington University, St. Louis, MO). Strain *nit1–305cw15* was obtained from P.A. Lefebvre (University of Minnesota, St. Paul) and has been used extensively as a nuclear-transformation recipient (Kindle et al., 1989; Kindle, 1990).

Plasmid DNAs and Nuclear Transformation

pγ contains the *AtpC* gene on a 3.8-kb *Sal*I fragment cloned into Bluescript SK (Smart and Selman, 1992). To facilitate construction of site-directed mutations, a subclone containing the 2.0-kb *Sal*I-*Sst*I fragment (Fig. 1) was constructed by digesting pγ with *Sst*I and self-ligating it. An *Eco*RI site was introduced 54 bp upstream of the initiation codon by site-directed mutagenesis (Kunkel et al., 1991) using the primer 5′-GCAAGTGAATTCTTGAACTGCGC. *Nhe*I restriction sites were individually introduced into three sites of the *C. reinhardtii* CF_1-γ TP, as illustrated in Figure 1, using the following oligonucleotides: 5′-CTATGCTCGCTAGCAAGCAGGG (Nhe-i), 5′-GGCGTC-

GCTAGCCGCGGCT (Nhe-c), and 5′-CCAGCCGCGCT-AGCCTGCAGGTG (Nhe-d). To place the mutated *Sal*I-*Sst*I fragments into the context of a full-length *AtpC* gene, they were subcloned into *Sal*I+*Sst*I-digested pγ1, which contains the region between the *Sal*I and downstream *Nco*I sites indicated in Figure 1. Plasmids containing the entire *AtpC* gene plus introduced restriction sites were named pγ-Eco (+*Eco*RI), pγ-Nhe-c (+*Eco*RI+Nhe-c), pγ-Nhe-d (+*Eco*RI+Nhe-d), and pγ-Nhe-i (+*Eco*RI+Nhe-i). To confirm that none of these restriction site modifications affected gene expression at the level of protein accumulation, they were individually transformed into CC-3022, and immunoblot analysis was performed as described below. The ATPase complex accumulated to the wild-type level in all cases (data not shown). To create deletions within the CF_1-γ TP-coding region, *Eco*RI-*Nhe*I fragments encoding the N-terminal part of the TP were subcloned into *Nhe*I-*Eco*RI fragments containing the C-terminal part of the TP, the remainder of the CF_1-γ-coding region, vector sequences, and the *AtpC* promoter. For example, the combination of the N-terminal *Eco*RI-*Nhe*I fragment from pγ-Nhe-i with the C-terminal *Nhe*I-*Eco*RI fragment from pγ-Nhe-c removed 20 amino acids between the *Nhe*I sites and was named pγ-Δ7–26.

PetE (PC) genes in which the stroma-targeting domain of the bipartite TP was replaced by the CF_1-γ TP were constructed as follows. Similarly to the strategy described above, an *Eco*RI site was introduced 28 bp upstream of the initiation codon, and *Nhe*I sites were engineered into Arg-Ser-coding sites in the PC TP (K.L. Kindle, unpublished data). *Nhe*I sites e and f lie on either side of the putative stroma-processing site between the predicted stroma-targeting and lumen-translocation domains. A series of chimeric *PetE* genes that were expressed from the *PetE* promoter was created by replacing *Eco*RI-*Nhe*I fragments from pPC-Nhe-e and pPC-Nhe-f with the corresponding fragments from pγ Nhe-c and pγ Nhe-d. A series of chimeric *PetE* genes driven by the *AtpC* promoter was created by replacing the *Kpn*I-*Nhe*I fragments in the pPC constructs with the corresponding fragments from the pγ plasmids. The names for these constructs include the fusion sites in the CF_1-γ and PC TPs. For example, γ26-PC20 contains 26 N-terminal amino acid residues from the CF_1-γ TP fused to the PC TP at amino acid residue 20.

To create chimeric genes in which the CF_1-γ TP-coding region was fused to that of the BRP (encoded by the *ble* gene), a *Pst*I site was introduced immediately upstream of the BRP initiation codon in pSP109 (V. Lumbreras and S. Purton, personal communication) by site-directed mutagenesis using the oligonucleotide 5′-CACTCAACATCT-GCAGATGGCCAAGCTG. The *Bam*HI-*Pst*I fragment of pγEco was cloned upstream of this *Pst*I site to create pγ-ble, which encodes a chimeric precursor protein in which 32 amino acid residues from the CF_1-γ TP are fused to the BRP immediately adjacent to the initiation codon. Deletion derivatives were created by replacing the *Kpn*I-*Pst*I fragment of pγ-ble with the corresponding fragments from pγ-Δ7–26, pγ-Δ7–29, and pγ-Δ27–29.

Mutant and wild-type *AtpC* genes were cotransformed into the nuclear genome of strain CC-3022 using the glass bead method (Kindle, 1990), with pMN24 DNA (Fernández et al., 1989), which contains the *Nit1* gene, for selection. Chimeric *PetE* genes were cotransformed into strain AP6, using *Bam*HI-digested pArg7.8 DNA for selection (Debuchy et al., 1989; K.L. Kindle, unpublished data). *Kpn*I-digested pSP109, pγ-ble, or pγ-ble TP-deletion derivatives were transformed into *nit1–305cw15* or CC-3396, and phleomycin-resistant transformants were selected on SGII-NH_4 or high-salt acetate plates with 2 to 5 μg/mL phleomycin (Stevens et al., 1996).

To produce CF_1-γ precursors for in vitro import assays, the *AtpC* cDNA insert of pTZ18R-γCF_1-1A (Yu et al., 1988) was cloned into the SP6 transcription vector pGEM7f$^+$ (Promega) to produce pγcD1. The wild-type and deleted TP-coding regions were cloned from the genomic context into pγcD1 in three steps. First, a 310-*Eco*RI-*Eag*I fragment from pγcD1, which includes the *Pst*I site in exon I, was subcloned into BluescriptII KS$^-$ to produce pγcD1-EE. Second, the *Eco*RI-*Pst*I fragment from pγcD1-EE was replaced by *Eco*RI-*Pst*I fragments from pγ-Eco, pγ-Δ27–29, pγ-Δ7–26, and pγ-Δ7–29. Third, the *Eco*RI-*Eag*I fragments from these plasmids, containing the wild-type and deleted genomic TP-coding regions, were then cloned back into *Eco*RI+Eag-digested pγcD1.

In Vitro Import Assay

Radiolabeled precursors were imported into isolated *C. reinhardtii* chloroplasts as described by Lawrence and Kindle (1997). CF_1-γ precursors were synthesized by in vitro transcription and translation in the presence of [^{35}S]Met using a rabbit reticulocyte lysate from Promega. *C. reinhardtii* chloroplasts were isolated, incubated for 0 to 20 min with labeled precursor that had been diluted 1:3 with 30 mM Met in import buffer, treated with thermolysin, and repurified through Percoll gradients. The proteins recovered from reisolated chloroplasts were dissolved in SDS sample buffer and fractionated in 12.5% SDS-polyacrylamide gels.

Chloroplast Isolation

Chloroplasts were isolated from *nit1–305cw15* transformants basically as described by Belknap (1983), with the following exceptions. We have found that walled cells comigrate with intact chloroplasts on Percoll gradients and, in some strains carrying the *cw15* mutation, a fairly large fraction of cells appear to contain residual cell wall material by this criterion. Therefore, *nit1–305cw15* cells were incubated in gamete lytic enzyme (prepared as described by Kindle, 1990) for 45 min at 37°C and then washed twice in 10 mM Hepes, pH 7.5. Wall-less cells were enriched by spinning twice through a layer of 70% Percoll prepared in BB (Belknap, 1983) plus 1% BSA. After the cells were washed in BB they were resuspended in BB plus 1% BSA and broken in an ice-cold Kontes press by incubation for 3 min at 35 p.s.i. and then separated in a 45/70% step Percoll gradient prepared in BB plus 1% BSA. The chloroplast fraction was collected from the 45/70% Percoll interface, diluted in BB, harvested, washed once in sorbitol

buffer (0.33 M sorbitol and 50 mM Hepes-KOH, pH 8.0), spun at 4000*g* for 1 min, and then subjected to immunoblot analysis, as described below.

DNA and Protein Blots

Transformants and the recipient strain were grown in SGII-NO_3 or SGII-NH_4 liquid medium, respectively, until late log phase (approximately 5×10^6/mL). DNA was prepared as previously described (Kindle et al., 1989). Following separation in 1% agarose gels, DNA was transferred to Hybond-N^+ (Amersham) by capillary blotting. After UV-cross-linking, the blot was hybridized with a probe made from the *AtpC* genomic *Bgl*II fragment (Fig. 1) by random priming (Feinberg and Vogelstein, 1983). Hybridization and washing were performed at 65°C, using the buffer described by Church and Gilbert (1984).

For immunoblot analysis, cells from 1.5-mL cultures were harvested by centrifugation and saved as pellets at −80°C until use. Cells were resuspended in SDS sample buffer and fractionated in 12% SDS-polyacrylamide gels (Stern et al., 1991). After equilibration in transfer buffer, proteins were transferred to nitrocellulose membranes using a Bio-Rad semidry electroblotter. Blots were blocked, incubated with antisera, and washed as previously described (Stern et al., 1991). Crude antisera raised against the indicated proteins were used at the following dilutions: *C. reinhardtii* CF_1-γ (from B. Selman), 1:1,000; spinach CF_1-β (from R. McCarty, Johns Hopkins University, Baltimore, MD), 1:100,000; *C. reinhardtii* PC (from S. Merchant, University of California, Los Angeles), 1:1,000; *Streptoalloteichus hindustanus* bleomycin-binding protein (from Cayla Sarl, Toulouse, France), 1:500; and *C. reinhardtii* Oee2, the 23-kD protein of the oxygen-evolving complex (from F.-A. Wollman, Institut de Biologie Physico-Chimique, Paris), 1:10,000. The identity of the CF_1-γ band was confirmed on blots not shown by using two antisera raised against spinach CF_1-γ (from A. Jagendorf and B. Baird, Cornell University, Ithaca, NY, and from R. McCarty and K.-H. Suess, Gatersleben, Germany). For detection via enhanced chemiluminescence, blots were incubated with Promega anti-rabbit IgG-conjugated horseradish peroxidase (1:2,000), washed, and treated with luminol and H_2O_2 (Durrant, 1990). X-ray film exposures were for 5 s to 2 min. To detect PC species, acetone-precipitated proteins were prepared and assayed by immunoblots as described by K.L. Kindle (unpublished data).

RESULTS

AtpC Genes That Have Large Deletions in the TP-Coding Region Complement the Leaky, Nonphotosynthetic Phenotype of *atpC1*

To assess TP function in CF_1-γ, we made several deletions in the 35-amino acid residue TP. As detailed in "Materials and Methods" and illustrated in Figure 1, we first introduced *Nhe*I sites into three locations of the CF_1-γ TP-coding region of *AtpC*. The *Nhe*I recognition site encodes Ala-Ser; therefore, two of these mutations were silent, and the third changed Gly-Ser to Ala-Ser. By combining restriction fragments upstream and downstream of the introduced *Nhe*I sites, we constructed deletions ranging in size from 3 to 23 amino acid residues, which were localized between residues 7 and 29. We first tested the effects of these mutations by transforming the mutant *AtpC* genes into a *C. reinhardtii* strain carrying the *atpC1* structural gene mutation (Smart and Selman, 1991a).

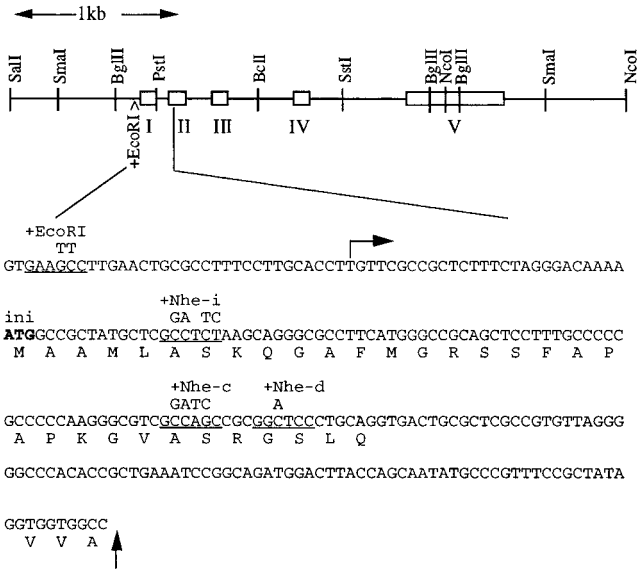

Figure 1. *AtpC* gene structure and locations of introduced restriction sites. A schematic representation of the *Sal*I-*Nco*I *AtpC* genomic DNA fragment used for transformation is shown at the top, with the sequence between an *Eco*RI site introduced 55 bp upstream of the initiation codon and the end of the TP-coding region shown below. Open boxes represent the five exons (I–V). The site of transcription initiation is indicated by a horizontal arrow (Yu et al., 1988; Quinn and Merchant, 1995). Stromal processing occurs at the end of the TP, as indicated by the vertical arrow (Yu and Selman, 1988). The initiation codon (ini) is shown in bold. The sites of introduced restriction sites are underlined in the wild-type sequence, with the nucleotide changes shown above. The sequence of the *Pst*I site used for the fusion to the *ble*-coding region is shown in italics.

As a transformation recipient, we used CC-3022 (*nit1atpC1*), which was constructed by Smart and Selman (1991a) by performing nuclear transformation in the presence of herring-sperm DNA. This apparently caused an insertion event that resulted in the arsenate-resistant, ATPase-deficient phenotype that exhibited leaky, nonphotosynthetic growth. For this reason and because we anticipated that TP mutations might impair import and accumulation of CF_1-γ, mutant and wild-type *AtpC* genes were introduced into the nuclear genome by cotransformation with the *Nit1* gene, which allows transformants to be selected on acetate-containing nitrate plates (SGII-NO_3).

In all transformations that included the wild-type *AtpC* gene or controls in which restriction sites were introduced upstream of or within the CF_1-γ-coding region, both large and small Nit^+ transformant colonies were recovered on selective plates. In the minus DNA control, no Nit^+ colonies were recovered, whereas with the *Nit1* gene alone, all

of the Nit$^+$ colonies were small. It seemed likely that the large Nit$^+$ colonies were transformants in which the *atpC* mutation had been complemented. All of the large colonies accumulated wild-type levels of chloroplast ATPase, whereas small colonies accumulated the same low level of ATPase seen in the parental strain (data not shown). This suggested an easy way to identify cotransformants that expressed a functional *AtpC* gene.

Surprisingly, CC-3022 cells that were cotransformed with *AtpC* genes containing TP-coding region deletions (pγ-Δ27–29, pγ-Δ7–26, and pγ-Δ7–29) also yielded small and large Nit$^+$ colonies. Whole-cell proteins were prepared from large Nit$^+$ colonies and analyzed for accumulation of chloroplast ATPase subunits. Figure 2 shows the immunoblot results for a representative pair of transformants from each mutant construct. Duplicate blots were reacted with antisera raised against spinach CF_1-β and *C. reinhardtii* CF_1-γ. The CF_1-β antiserum reacted with both mitochondrial F_1-β, an indication of the amount of whole-cell protein loaded into each well, and chloroplast CF_1-β, which accumulates in proportion to the total amount of chloroplast F_1. Figure 2 shows that all transformants accumulated significantly more CF_1-β and CF_1-γ than the *atpC1* recipient (CC-3022). Furthermore, the levels of CF_1-β and CF_1-γ were similar in all transformants. The CF_1-γ protein that accumulated in Δ7–29 transformants migrated slightly behind wild-type CF_1-γ, suggesting that the precursors in these transformants were processed aberrantly or not at all.

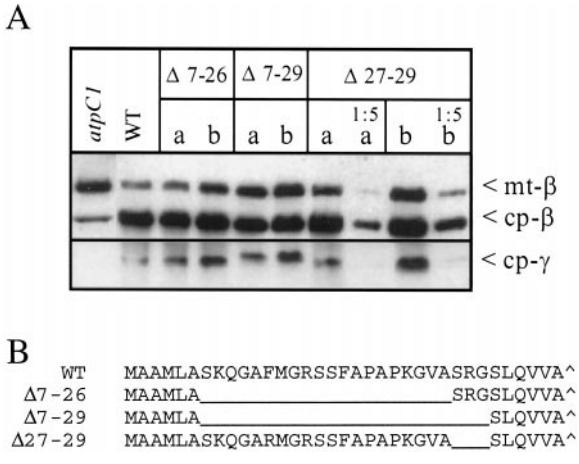

Figure 2. Nit$^+$ colonies cotransformed with *AtpC* genes carrying TP-deletion mutations accumulate wild-type levels of ATPase subunits. A, Immunoblot. Whole-cell proteins were isolated from the *atpC1* transformation recipient transformed only with *Nit1* (*atpC1*) or robust Nit$^+$ transformants that were cotransformed with the wild-type *AtpC* gene (WT) or *AtpC* genes with TP deletions (Δ). Proteins were fractionated in a 12% SDS-polyacrylamide gel and transferred to nitrocellulose, and immunoblot analysis was performed as described in "Materials and Methods." The upper blot was incubated with antiserum raised against spinach CF_1-β; both mitochondrial (mt-β) and chloroplast (cp-β) species were detected. The lower blot shows only the CF_1-γ region of the blot that was incubated with antiserum raised against *C. reinhardtii* CF_1-γ (cp-γ). B, Sequences of wild-type and deleted TPs; the extent and location of the deleted amino acid residues are indicated with a line.

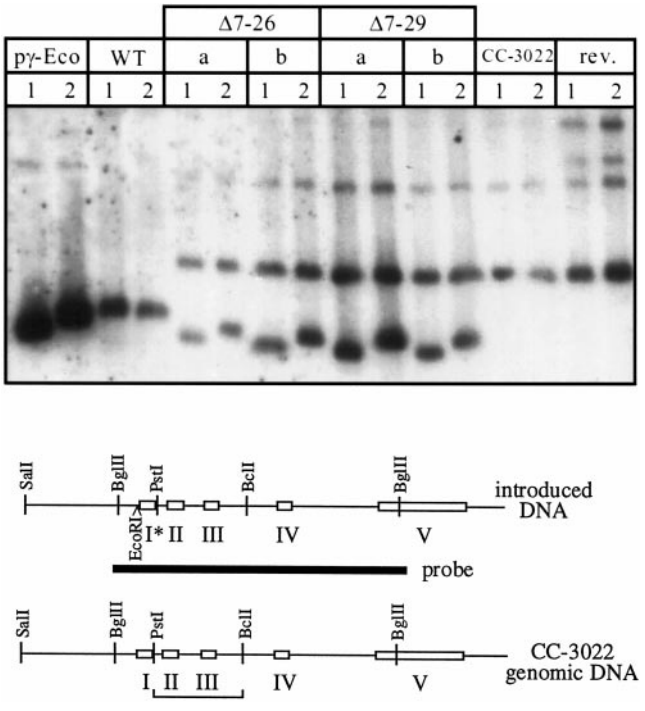

Figure 3. Transformants that accumulate CF_1-γ contain an ectopic copy of the introduced *AtpC* gene. Whole-cell DNA was prepared from the CC-3022 strain, phenotypic revertants (rev.), the wild-type P17 strain (WT), or robust Nit$^+$ transformants that had been cotransformed with *AtpC* deletion constructs (Δ7–26 and Δ7–29). *C. reinhardtii* DNA or a plasmid containing an introduced *Eco*RI site in the *Sal*I-*Nco*I fragment (pγ-Eco) was digested with *Bgl*II plus *Eco*RI (lanes 1) or *Bgl*II only (lanes 2). The DNAs were separated in an agarose gel, transferred to a nylon filter, and hybridized with the *Bgl*II fragment, which is indicated as a heavy line between the restriction maps. The *Eco*RI site in the introduced DNA is indicated on the upper map; symbols are as defined for Figure 1. The asterisk indicates that the TP deletions are in exon I. The horizontal bracket below the lower map indicates that a DNA rearrangement has occurred in the *Pst*I-*Bcl*I fragment of CC-3022.

Transformants Contain an Ectopic Copy of the Introduced *AtpC* Gene

The accumulation of a higher-molecular-weight CF_1-γ protein in Δ7–29 transformants argued strongly against the possibility that a reversion or homologous recombination event within the endogenous *AtpC* gene had generated a wild-type TP that allowed accumulation of CF_1-γ. Nonetheless, we wanted to confirm that the large Nit$^+$ transformants contained additional *AtpC* gene copies that had integrated outside of the *AtpC* locus. Therefore, we prepared whole-cell DNA from CC-3022, a wild-type strain (P17), the Δ7–26 and Δ7–29 transformants shown in Figure 2, and a phenotypic revertant of CC-3022 (see "Materials and Methods"). DNA was digested with restriction enzymes and hybridized with the *Bgl*II fragment labeled "probe" in Figure 3. We had previously mapped a restriction fragment length polymorphism in CC-3022, presumably the consequence of the insertion of herring-sperm DNA, to the *Pst*I-*Bcl*I fragment that contains exons II and III (data not shown). Figure 3 shows that the size of the

AtpC *Bgl*II fragment in CC-3022 was significantly larger than the corresponding fragment in the wild type. To distinguish endogenous *AtpC* genes from the introduced ones, we took advantage of the additional *Eco*RI site in the introduced genes. Plasmid DNA containing the upstream *Eco*RI site in *AtpC* (pγ-Eco) showed the expected size difference in *Bgl*II versus *Bgl*II plus *Eco*RI-digested *AtpC* fragments (Fig. 3). Whole-cell DNA preparations from Δ7–26 and Δ7–29 transformants contained the higher-molecular-weight *Bgl*II fragment characteristic of CC-3022 DNA, as well as an additional fragment of about the same intensity that contained the *Eco*RI site diagnostic of introduced genes. As expected, the *Bgl*II and *Bgl*II-*Eco*RI fragments from Δ7–26 and Δ7–29 whole-cell DNA ran slightly ahead of those in pγ-Eco, which do not carry the TP-coding region deletions. These results are consistent with ectopic integration of the transforming DNA and inconsistent with a homologous recombination event at the *AtpC* locus. Consequently, the most likely explanation for the accumulation of wild-type levels of CF_1-γ is that import of the mutant precursor occurs at a rate high enough that it does not limit ATPase accumulation.

It was previously reported that *atpC1* is a stable, null mutation (Smart and Selman, 1991a). However, in our hands strain CC-3022 grew slowly on medium lacking acetate, and colonies with a more robust photoautotrophic phenotype appeared at a low frequency (10^{-7}). To further characterize the recipient strain and to determine the amount of CF_1-γ that is required for a photoautotrophic phenotype, whole-cell proteins from the mutant and revertant strains were analyzed for accumulation of CF_1. Figure 4 shows that two photosynthetic revertants of CC-3022 accumulated about 10% of the wild-type level of CF_1-β and CF_1-γ; this is about twice the amount of CF_1-β observed in CC-3022. CF_1-γ abundance was below the level of detection in CC-3022. We wondered whether there had been an excision of part or all of the introduced DNA that accounted for the enhanced level of protein accumulation in the phenotypic revertants. However, as shown in Figure 3 (and data not shown), the *Bgl*II fragment characteristic of CC-3022 is still present in the revertant strains. It is interesting that all of the tested revertants contained a higher-molecular-weight fragment that hybridized weakly with the *AtpC* probe.

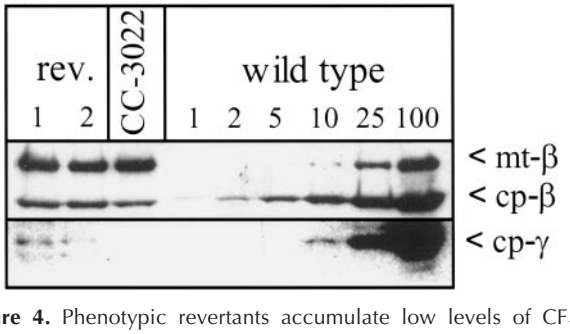

Figure 4. Phenotypic revertants accumulate low levels of CF_1-γ. Whole-cell proteins from CC-3022, two photosynthetic revertants of CC-3022 (rev. 1 and 2), and the P17 wild-type strain were subjected to immunoblot analysis, as described in the legend to Figure 2. In the wild-type lanes, proteins loaded were equivalent to 1, 2, 5, 10, 25, and 100% of the amount loaded into the first three lanes.

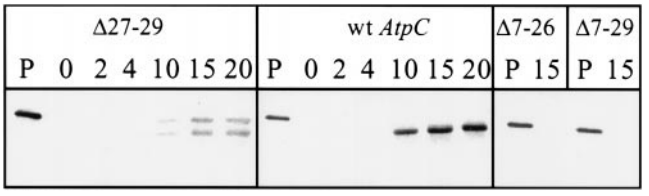

Figure 5. Deletions in the CF_1-γ TP impair in vitro import. ^{35}S-labeled precursors containing the wild-type CF_1-γ TP (wt *AtpC*), or TP deletions between the designated amino acid residues were synthesized and incubated for the indicated times (in minutes) with isolated *C. reinhardtii* chloroplasts in the presence of 10 mM ATP, as described in "Materials and Methods." Following import, chloroplasts were treated with thermolysin and repurified, and proteins were separated in a 12.5% SDS-polyacrylamide gel. P, five percent of the in vitro translated precursor that was used for the import assays.

In Vitro Import Is Undetectable for Precursors with TP Deletions Δ7–26 or Δ7–29

The accumulation of wild-type levels of chloroplast ATPase in transformants expressing the deleted *AtpC* genes was unexpected, since in vitro chloroplast protein import assays in vascular plants and *C. reinhardtii* have suggested that only fairly short TP deletions are tolerated without severely impairing import. Therefore, it was important to determine whether the CF_1-γ TP deletions affected import using a homologous in vitro chloroplast protein-import assay. To do this, the deletions that had been introduced into the *AtpC* gene were subcloned into the context of *AtpC* cDNA behind an SP6 promoter. As a control, the wild-type genomic sequence was also placed in this context; the only difference between the wild-type genomic and cDNA upstream sequences is that the introduced *Eco*RI site in the genomic sequence is about 30 bp upstream of the normal 5′ end of the transcript, so the in vitro transcript is correspondingly longer.

We recently established a homologous *C. reinhardtii* chloroplast protein-import assay (Goldschmidt-Clermont et al., 1989) and demonstrated that import is time and energy dependent (Lawrence and Kindle, 1997). As shown in Figure 5, when wild-type or Δ27–29 CF_1-γ precursor was added to the assay, a lower-molecular-weight, protease-protected species, which presumably represents imported and processed CF_1-γ, was detected after 10 min. It is interesting that a higher-molecular-weight, protease-resistant species was also detected in the assay with the Δ27–29 precursor, suggesting that import or processing may be partially impaired by this deletion. In contrast, no processed or protease-protected CF_1-γ precursor was detected for the Δ7–26 or Δ7–29 precursor after 15 min, a time when import of wild-type precursor had nearly reached a maximum. This suggests strongly that these deleted TPs do not allow significant precursor import in vitro. However, it should be noted that this in vitro import assay was capable of detecting only about 5% of the wild-type level of PC

import (Lawrence and Kindle, 1997); therefore, inefficient import of CF_1-γ might not have been detected.

The CF_1-γ TP Is Sufficient for in Vivo Stroma Targeting

Because at least two-thirds of the CF_1-γ TP could be deleted without affecting accumulation of CF_1-γ in vivo, we wondered whether it contained in vivo stroma-targeting information. Therefore, constructs were designed to determine whether the CF_1-γ TP could restore stroma-targeting function to a defective PC TP. PC is a thylakoid lumen-localized protein with a bipartite TP (Smeekens et al., 1986; Merchant et al., 1990; Lawrence and Kindle, 1997). Deletions near the N terminus of the stroma-targeting domain of the PC TP eliminate in vivo PC accumulation almost completely (Fig. 6; K.L. Kindle, unpublished data). Since the PC-encoding *PetE* transcript accumulates and is translated in these transformants, and the precursor half-life is significantly longer than that of the wild-type PC precursor, these N-terminally deleted TPs are most likely nonfunctional in chloroplast protein import. Therefore, to determine whether the CF_1-γ TP could restore stroma-targeting function to a defective PC precursor, we fused 26 or 29 amino acids of the CF_1-γ TP to two different places in the bipartite PC TP, either immediately N-terminal or C-terminal to the putative stromal protease-processing site.

Figure 6, B and D, shows the maps and sequences of chimeric TPs fused to the PC-coding region, with gene expression driven by either the *AtpC* or *PetE* promoter. These constructs were cotransformed into strain AP6 *(arg7pcy1–1 mt$^+$)*, which carries two mutations in the PC-coding region that cause a frameshift resulting in premature translation termination (Quinn et al., 1993); the *Arg7* gene was used for selection. Twenty Arg^+ transformants from each construct were screened for PC accumulation by immunoblot analysis. The immunoblot in Figure 6E illustrates the highest level of PC accumulation observed among transformants generated with each of the eight constructs. In transformants carrying constructs in which the CF_1-γ TP-coding region was fused to the PC TP N-terminal of the putative stroma-processing site, PC accumulated to nearly the same level as in wild-type cells.

Mature PC also accumulated in transformants expressing the γ26–30PC construct, in which the precursor contained 26 amino acid residues from the CF_1-γ TP-fused C-terminal of the processing site, at position 30 in the PC TP. There was no significant difference in PC accumulation, whether the constructs were expressed from the *PetE* or *AtpC* promoter. Since PC did not accumulate to a significant level in PC TP mutants lacking N-terminal amino acids (Δ2–8 and Δ2–18 in Fig. 6), the stroma-targeting function in the chimeric constructs was presumably provided by the CF_1-γ TP. In contrast, transformants that expressed the fusion of the longer CF_1-γ TP region to the C-terminal site in the PC TP (γ29–30PC) accumulated barely detectable levels of PC. Moreover, this species migrated slightly more slowly than wild-type, mature PC, suggesting that it was aberrantly processed. These results indicate that the *AtpC* TP can have an in vivo stroma-targeting function, but that

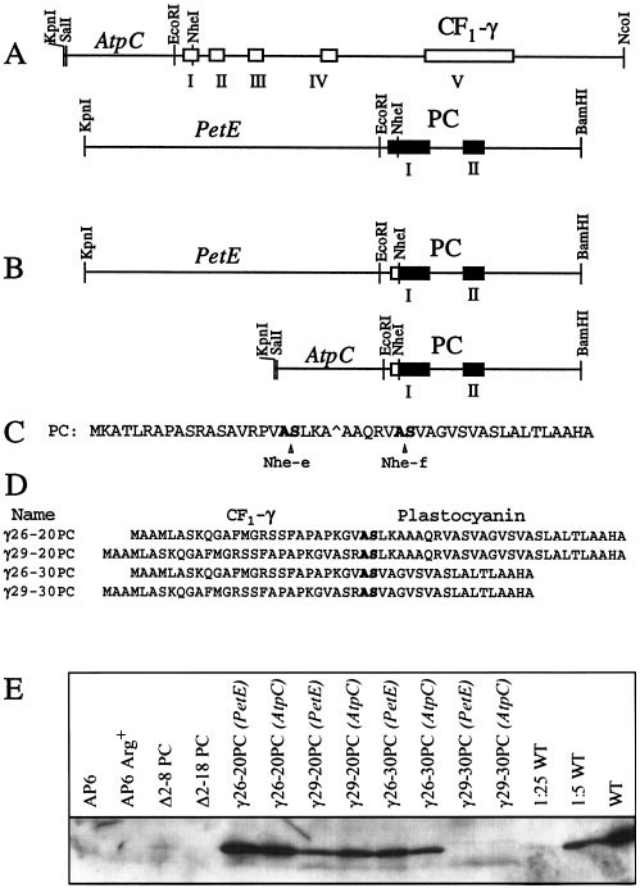

Figure 6. A, Diagram of the genomic *AtpC* and *PetE* genes; coding regions are shown as open and closed boxes, respectively. B, Maps of constructs that use either the *PetE* promoter (top) or the *AtpC* promoter (bottom) to drive expression of genes that contain a chimeric TP-coding region linked to the PC-coding region from *PetE*. C, The sequence of the wild-type PC TP. The two sites used for fusion to the CF_1-γ TP are shown in bold (labeled below as Nhe-e and Nhe-f), and the postulated location of the stromal protease-processing site is indicated by an arrowhead. D, Names and amino acid sequences of the four chimeric TPs; the fusion sites are shown in bold. E, Accumulation of PC in Arg^+ transformants. Whole-cell protein extracts were isolated from the transformation recipient (AP6), from an Arg^+ transformant, and from Arg^+ transformants that expressed the indicated *PetE* genes (the promoter is indicated in parentheses). Proteins were separated in a 12 to 18% gradient SDS-polyacrylamide gel and subjected to immunoblot analysis with antiserum raised against *C. reinhardtii* PC. 1:25, 1:5, and undiluted proteins from a transformant containing the wild-type (WT) *PetE* gene are shown in the three right lanes.

the functionality of the chimeric TP depends on the details of the fusion.

Deletions in the CF_1-γ TP Impair Its in Vivo Stroma-Targeting Function

The experiment described above demonstrated that the CF_1-γ TP has in vivo stroma-targeting function in the context of a PC fusion protein. However, it was still important to test whether the deletions that prevented in vitro chlo-

roplast protein import affected in vivo import, as inferred from the accumulation and subcellular localization of chimeric proteins. Because we were also interested in characterizing a marker that might be useful for selecting mutants defective in chloroplast protein import, we fused wild-type and deleted CF_1-γ TP-coding regions to the *Streptoalloteichus hindustanus ble* gene, which confers resistance to the DNA-damaging agents phleomycin and bleomycin (Stevens et al., 1996). The *ble* gene encodes a BRP that binds the drug stoichiometrically (Dumas et al., 1994). Since the primary target of DNA-damaging agents is presumably the nucleus, a protein localized to the chloroplast might not confer drug resistance. In fact, phleomycin-resistant transformants were recovered with all constructs, although the number of resistant colonies was consistently lower with pγ-ble, which contains a wild-type CF_1-γ TP, than with pSP109 or with constructs that encoded chimeric proteins with TP deletions (Table I; data not shown). As discussed further below, this marker may be useful for selecting import-defective mutants.

To determine whether the wild-type or mutant TPs caused the BRP to be associated with chloroplasts, plastids were isolated from transformants that expressed the protein at a high level in preliminary experiments, and immunoreactive proteins were compared with those from whole cells by performing immunoblots. All transformants accumulated an immunoreactive protein with an electrophoretic mobility consistent with the predicted molecular mass of the unprocessed BRP precursor (between 14 and 17.5 kD, depending on the TP, indicated by asterisks in Fig. 7), although the amount was somewhat less for the Δ7–26 and Δ7–29 transformants than for the wild-type and Δ27–29 constructs. In the case of the wild-type CF1-γ TP, approximately 25% of the total fusion protein was associated with the chloroplast fraction, which was enriched in a more rapidly migrating polypeptide that may represent a processed species (indicated by a bullet in Figure 7).

A small amount of the total Δ27–29 CF1-γ-BRP was associated with the chloroplast fraction, where two distinct species were visible. The overexposed panel shows a trace amount of immunoreactive material in the chloroplast fraction of transformants expressing the Δ7–26 CF1-γ-BRP, whereas none of the BRP lacking a TP or Δ7–29 CF1-γ-BRP appeared to be chloroplast localized. These results are consistent with the suggestion that 32 amino acid residues from the CF1-γ TP are sufficient to direct a foreign protein into *C. reinhardtii* chloroplasts in vivo. Since cytosolic BRP fusion proteins accumulated in transformants containing chimeric genes with TP-coding region deletions, but little if any protein was chloroplast associated, the simplest explanation is that these TP deletions significantly impaired import of the fusion protein. However, we cannot eliminate the possibility that these fusion proteins were imported into the chloroplast but rapidly degraded.

DISCUSSION

We have analyzed the effects of TP deletions on import of the extrinsic thylakoid membrane protein CF_1-γ in *C. reinhardtii.* Import was assessed in two ways: (a) in a homologous in vitro import assay and (b) by accumulation of the protein in transformants that express the mutant genes. Very different results were obtained with the two approaches. Precursors lacking two-thirds of the TP failed to be imported to a detectable level in vitro, but transformants that expressed the corresponding mutant *AtpC* genes in vivo accumulated wild-type levels of ATPase subunits.

We were initially concerned that photosynthetic colonies might have arisen from reversion of the original mutation or that wild-type *AtpC* genes might have been generated by homologous recombination (Sodeinde and Kindle, 1993). However, large (photosynthetic) Nit^+ colonies were recovered only when *AtpC* constructs were included in the transformation experiments. Furthermore, analysis of genomic DNAs suggested that only ectopic integration events had occurred, since the endogenous *AtpC* locus was unaltered. Finally, accumulation of a higher-molecular-weight CF_1-γ species in the Δ7–29 transformants suggested that this species was the product of the introduced gene.

The CF_1-γ TP

Although it is certainly possible that there are fundamental differences in TP requirements for in vivo and in vitro

Table I. *Recovery of phleomycin-resistant transformants*

Glass bead transformation was carried out with 2 μg of the indicated DNA and 4×10^7 cells (Kindle, 1990). Cells grew in acetate-containing liquid medium for 18 h following transformation, and cultures were split in half and plated on high-salt acetate (CC-3396) or SGII-NH_4 (*nit1-305cw15*) plates containing 2 or 5 μg/mL phleomycin (Phl).

Construct	*nit1-305cw15*		CC-3396		
	*Kpn*I-digested DNA		*Kpn*I-digested DNA		Supercoiled DNA
	2 μg/mL Phl	5 μg/mL Phl	2 μg/mL Phl	5 μg/mL Phl	5 μg/mL Phl
pSP109	13	13	15	7	4
pγ-ble	7	4	8	1	0
pγ-ble Δ7-26	35	30	30	4	5
pγ-ble Δ7-29	15	8	4	1	6
pγ-ble Δ27-29	17	10	17	24	4
−DNA	0	0	0	0	0

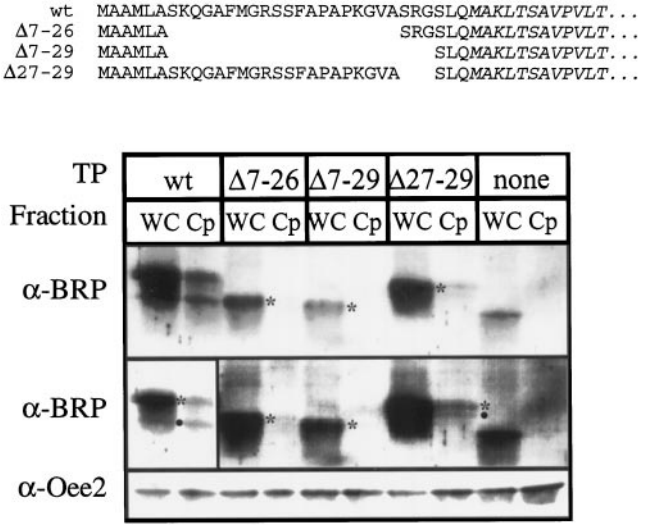

Figure 7. Deletions in the CF_1-γ TP prevent the accumulation of chloroplast-localized BRPs. The sequences of fusions between wild-type (wt) or deleted CF_1-γ TPs and the BRP are shown at the top, with the TP sequence in plain text and the N-terminal sequence of the BRP in italics. Transformants expressing the various fusion proteins were analyzed in immunoblots. Proteins from whole cells (WC) or chloroplasts (Cp) isolated as described in "Materials and Methods" were denatured in sample buffer and fractionated in a 15% SDS-polyacrylamide gel, transferred to nitrocellulose, and reacted with antiserum raised against BRP or Oee2, the 23-kD polypeptide of the oxygen-evolving complex, which is localized in the chloroplast lumen. In the top and bottom panels, all lanes were exposed equally. The middle panel shows a shorter exposure of the wild-type lanes, and a longer exposure of the remaining lanes, to more clearly demonstrate the level of chloroplast-associated BRP. Putative chimeric precursors are indicated by asterisks, and processed species are indicated by bullets.

import, this seems unlikely. Two lines of evidence indicate that the CF_1-γ TP has in vivo stroma-targeting activity. First, when the putative PC stroma-targeting domain was replaced by the CF_1-γ TP, PC accumulated to nearly the wild-type level in most cases, suggesting that import had been restored. Second, when the CF_1-γ TP-coding region was fused to the *ble* gene and transformed into *C. reinhardtii*, about 25% of the immunoreactive BRP appeared to be associated with the chloroplast fraction. Two protein species were detected, and the smaller one was enriched in chloroplasts, suggesting that it may represent a stroma-processing product. Since the wild-type fusion proteins contained 32 of the 35 amino acids of the CF_1-γ TP, the normal CF_1-γ-processing site was not present. Although the cleavage site in the fusion protein has not been determined, its electrophoretic mobility is consistent with processing close to the junction between the TP and the BRP.

When TP deletions that had no effect on in vivo CF_1-γ accumulation were introduced into the context of the BRP, they appeared to eliminate in vivo chloroplast protein import partially or nearly completely, since little or none of the fusion protein that accumulated was chloroplast associated. Although we cannot eliminate the possibility that fusion proteins with deletions in the TP are imported into the chloroplast and then rapidly degraded, this seems unlikely. Fusion proteins with TP deletions do accumulate in the cytosol; therefore, their lability would have to be chloroplast specific. Furthermore, the Δ27–29 species, which was detectable in the chloroplast fraction only at a low level, differed by only three amino acids from the wild-type fusion protein, which accumulated to a relatively high level in the chloroplast. The simplest interpretation is that the in vivo chloroplast-targeting function of the CF_1-γ TP is impaired by these deletions, in some cases severely. It is possible that redundant stroma-targeting information resides within the mature CF_1-γ protein and accounts for import and accumulation of CF_1-γ protein in vivo, but if so, this information is recognized only in vivo. We favor the interpretation that import of CF_1-γ is impaired even in vivo by the long TP deletions, but that relatively little import is sufficient to support wild-type levels of ATPase complex assembly.

The Δ7–29 TP deletion either prevents in vivo processing of the CF_1-γ precursor or causes aberrant processing, since a higher-molecular-weight CF_1-γ species accumulated in transformants carrying this mutation. Because of the large size of the deletion in Δ7–29, the precursor would be only 12 amino acid residues longer than the correctly processed mature protein. Apparently, the aberrant CF_1-γ species is assembled and functional in the ATPase complex, since the strain had a robust photosynthetic growth phenotype. Transformants expressing either the Δ7–26 or Δ27–29 precursors accumulated processed CF_1-γ. However, when the Δ27–29 CF_1-γ precursor was imported into isolated chloroplasts in vitro, a protease-protected precursor species was observed in addition to mature, processed CF_1-γ. Since the ratio of the two forms was constant during the import reaction, we speculate that the protease-protected precursor may represent a population that has entered a nonproductive import pathway and cannot be translocated across the inner envelope, or has adopted a conformation that is resistant to stromal processing. The aberrant processing of the Δ7–29 and Δ27–29 precursors in vivo and in vitro, respectively, is consistent with previously published studies in which mutations near the cleavage site affected the extent and/or fidelity of processing (Wasmann et al., 1988; Ostrem et al., 1989; Archer and Keegstra, 1993; Bassham et al., 1994; Pilon et al., 1995). A positively charged amino acid residue at position −4 relative to the cleavage site was required for processing of the soybean and pea light-harvesting chlorophyll *a/b*-binding protein precursors (Clark and Lamppa, 1991). In this respect, it is interesting to note that an Arg residue is present at −8 in the wild-type and Δ7–26 CF_1-γ precursors, whereas a Lys residue is present at position −10 in the Δ27–29 precursor. There are no positively charged residues in the Δ7–29 TP, which is probably unprocessed in vivo.

Accumulation of the Chloroplast ATPase

The constituent polypeptides of the thylakoid ATPase do not generally accumulate as unassembled proteins (Merchant and Selman, 1984). Consequently, their steady-state levels are determined by the synthesis, maturation, or as-

sembly of the rate-limiting subunit in the complex. Our results show that TP deletions that reduce in vitro import of the CF_1-γ precursor below the level of detection and that have a severe impact on accumulation of chloroplast-localized BRP fusion proteins in vivo have no significant effect on accumulation of CF_1-γ. This suggests that import of CF_1-γ does not limit accumulation of the chloroplast ATPase even though it may be severely impaired. This is consistent with the observation that *C. reinhardtii* chloroplast ATPase is extremely stable (Merchant and Selman, 1984), suggesting that relatively little new synthesis is required for accumulation of the complex to the wild-type level.

In this study the *atpC1* recipient strain (CC-3022) had a leaky nonphotosynthetic growth phenotype and accumulated CF_1-β to about 5% of the wild-type level. Although CF_1-γ was not detected in CC-3022 using three different antisera, these antisera also failed to detect 5% of the wild-type level of CF_1-γ (Fig. 4; data not shown). Although unassembled subunits of ATPase complexes do not generally accumulate, a *C. reinhardtii atpA* translation mutant (F54) accumulates some CF_1-β in the absence of CF_1-α, partially due to 3-fold increased synthesis of CF_1-β in this context (Drapier et al., 1992). Since CF_1-β and CF_1-γ accumulated in parallel in the phenotypic revertants described here, the simplest interpretation is that there is enough residual expression of *AtpC* in CC-3022 to allow the ATPase complex to accumulate to about 5% of the wild-type level. DNA-blot analysis was consistent with an alteration in the *Pst*I-*Bcl*I fragment that contains exons II and III, introns I and II, and part of intron III. We presume that an insertion of herring-sperm carrier DNA occurred in one of the introns during the course of nuclear transformation and that splicing is therefore impaired. However, a more complex rearrangement is also possible, since insertional mutagenesis sometimes results in deletions at the insertion site (Tam and Lefebvre, 1993.) The nature of the phenotypic reversion is unknown, since the original DNA rearrangement in *atpC1* was still present in both revertants tested. Perhaps a slight increase in splicing efficiency due to an extragenic suppressor mutation could account for the small (approximately 2-fold) increase in gene expression.

Strategies for Isolating Envelope Translocation Mutants

It is likely that some level of chloroplast protein import is required for cell viability. A single envelope translocation pathway appears to be utilized by most chloroplast precursors, and chloroplasts carry out essential metabolic functions in addition to photosynthesis, which probably require nucleus-encoded polypeptides. Consequently, the isolation of import-defective mutants may require screening of temperature-sensitive lethal mutants or selection schemes that can detect mutations that impair but do not abolish import. The BRP would appear to have potential as a selectable marker for envelope-translocation mutants, since it binds the drug stoichiometrically and may confer resistance only when localized in the cytosol. Defective localization of a chloroplast-targeted BRP in a phleomycin-sensitive strain might result in a phleomycin-resistant phenotype.

Preliminary results with *AtpC-ble* fusion genes and similar constructs with the PC TP (data not shown) suggest that BRP function is not impaired by N-terminally fused oligopeptides, since phleomycin-resistant transformants could be selected in most cases. Because the transformants characterized in this work were selected for phleomycin resistance, it is not surprising that some of the fusion protein remains cytosolic. The amount of cytosolic BRP may determine the level of phleomycin resistance, and this is likely to be a consequence of the level of gene expression as well as the efficiency of chloroplast import. The recovery of fewer phleomycin-resistant transformants with the wild-type TP fusions than with most TP-deletion constructs may indicate that a higher level of gene expression is required to recover phleomycin-resistant transformants carrying the wild-type construct, since the fusion protein can be imported into the chloroplast. We are screening for phleomycin-sensitive transformants that express the *ble* gene and accumulate processed BRP in the chloroplast, which will be used as the starting strains for selecting envelope translocation mutants.

Concluding Remarks

Although most of our understanding of the chloroplast protein-import process has been derived from elegant in vitro chloroplast protein-import experiments, it is unlikely that the complexity of the cellular milieu is fully reproduced in vitro. *C. reinhardtii* offers great potential as an experimental organism in which to study chloroplast protein import using in vivo and genetic approaches. However, in interpreting the in vivo consequences of TP mutations, one must consider the many processes that can regulate expression of the introduced gene and accumulation of the mature protein product. The disparate results obtained when mutant or chimeric gene products are assessed in vivo and in vitro may not reflect inherent differences in import processes so much as the additional complexity of the cellular environment.

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