Tip Loci: Six Chlamydomonas Nuclear Suppressors That Permit the Translocation of Proteins with Mutant Thylakoid Signal Sequences

Karen K. Bernd and Bruce D. Kohorn

Developmental, Cell and Molecular Biology Group, Department of Botany, Duke University, Durham, North Carolina 27708
Manuscript received January 20, 1998
Accepted for publication March 19, 1998

ABSTRACT

Mutations within the signal sequence of cytochrome f (cytf) in Chlamydomonas inhibit thylakoid membrane protein translocation and render cells nonphotosynthetic. Twenty-seven suppressors of the mutant signal sequences were selected for their ability to restore photoautotrophic growth and these describe six nuclear loci named tip1 through 6 for thylakoid insertion protein. The tip mutations restore the translocation of cytf and are not allele specific, as they suppress a number of different cytf signal sequence mutations. Tip5 and 2 may act early in cytf translocation, while Tip1, 3, 4, and 6 are engaged later. The tip mutations have no phenotype in the absence of a signal sequence mutation and there is genetic interaction between tip4, and tip5 suggesting an interaction of their encoded proteins. As there is overlap in the energetic, biochemical and genetic requirements for the translocation of nuclear and chloroplast-encoded thylakoid proteins, the tip mutations likely identify components of a general thylakoid protein translocation apparatus.

The translocation of proteins into and across membranes is an essential process that can occur either co- or posttranslationally. The mechanisms appear to involve signals within the translocated protein itself that are recognized by soluble and membrane factors that mediate the association with and insertion into the lipid bilayer (Schatz and Dobberstein 1996). Genetic and biochemical data show that a single membrane often uses mechanisms conserved between evolutionarily distant species and may use multiple translocation pathways. The thylakoid membrane system of chloroplasts contains at least three distinct mechanisms that effect translocation (Cline and Henry 1996). One is reminiscent of the bacterial Sec system, and a second contains characteristics that have not been described in other systems, as it does not involve nucleotide triphosphates or soluble factors, but rather requires a membrane ΔpH and thylakoid membranes (Cline et al. 1992). The third shows characteristics of the SRP-mediated translocation system first described for the endoplasmic reticulum (Cline and Henry 1996).

Studies with isolated pea chloroplasts have defined the energetic requirements for translocation of nuclear encoded proteins. All of the precursors reported require components of the thylakoid membrane for translocation. In addition to thylakoid membranes, plastocyanin (PC) requires SecA and ATP, the oxygen-evolving complex 23- and 17-kD proteins (OE23 and 17), the photosystem I N-subunit protein requires a ΔpH, and the light harvesting chlorophyll a/b binding protein (LHCP) requires a stromal protein, GTP and a ΔpH (Kirwin et al. 1989; Mould and Robinson 1991; Cline et al. 1992; Hulford et al. 1994; Nielsen et al. 1994; Yuan and Cline 1994; Kouranov and Schnell 1996). The stromal protein required by LHCP is likely to be CP54, a homologue of the SRP 54-kD protein, as its depletion from stroma ablates insertion (Li et al. 1995).

Studies of the integral membrane protein CF, II found that nucleoside triphosphates and stromal extracts are not required for its integration into the membrane, and that a ΔpH only slightly enhances its integration (Michl et al. 1994). Thus CF, II may engage components similar to those used by OE23 and 17 or these energetic requirements may be indicative of another translocation mechanism.

Homologues of bacterial SecA and SecY (Schatz and Dobberstein 1996), have been identified in vascular plant and algal chloroplasts (Nakai et al. 1992, 1994; Laird et al. 1995). Since SecY of bacterial membranes and its mammalian homologue, Sec61p of the ER, are thought to be protein translocases, it is assumed that SecY of thylakoids has a similar function. A requirement for SecA in PC translocation has been shown in isolated pea chloroplasts (Nakai et al. 1994; Yuan and Cline 1994). The th1 mutation of maize was found to be in the chloroplast SecA homologue and results in impaired translocation of a number of thylakoid proteins including PC and cytochrome f (cytf; Voelker et al. 1997). The decrease in the steady state levels of these proteins indicates that these proteins likely require SecA in vivo. The energetic requirements of thylakoid lumen pro-
teins, like PC and OE33, appear to be dependent upon the thylakoid transfer signal and not the mature portion of the protein, as exchange of signals also exchanges the energetic requirement (Cline et al. 1992; Robinson et al. 1994). This is not always the rule as LHCOP does not have a simple cleaved thylakoid signal sequence (Auchincloss et al. 1992; Huang et al. 1992) and requires multiple transmembrane helices for correct insertion. Collectively, the energetic data have been interpreted to indicate the presence of multiple mechanisms of translocation across the thylakoid. However, the results are also consistent with there being a variety of requirements necessary for different precursors to interact with just one apparatus. The latter view is supported by the results of in vivo studies of the translocation of Chlamydomonas cyt into the thylakoid membrane (Smith and Kohorn 1994). Mutations within the 31-amino-acid signal sequence of plastocyanin encoded cyt reduce or eliminate its translocation causing the cells to become nonphotosynthetic. Two point mutations (A15E and V16D) and a deletion of the signal sequence’s predicted hydrophobic core (hc) had the most pronounced effects and inhibited translocation into the lipid bilayer. Other signal sequence mutations helped delineate the boundaries of the essential hydrophobic core region (Bailliet and Kohorn 1996; Smith and Kohorn 1994). The signal sequence mutation cyt A15E not only inhibited the insertion of cyt but also decreased the accumulation of other integral thylakoid membrane proteins, suggesting that multiple precursors utilize the same apparatus. Recent biochemical analysis indeed finds that both LHCOP and cyt associate with CP54 in vitro (High et al. 1997). Thus while the energetic requirements differ between precursors, some may share protein translocation components (Smith and Kohorn 1994).

To gain an understanding of thylakoid translocation in vivo we developed a selection scheme in the haploid alga Chlamydomonas that was aimed at identifying proteins that are directly involved in the process. Point and deletion mutants of the cyt signal sequence were unable to translocate cyt into the thylakoid membrane, and resulted in strains with impaired ability to grow photosynthetically (Smith and Kohorn 1994). Twenty-seven strains that suppressed the cyt signal sequence alterations were selected by their ability to restore photosynthetic growth (Smith and Kohorn 1994). Four of these suppressor strains contained second site mutations in the cyt signal sequence and described an important hydrophobic core whose position and exact amino acid composition were not critical (Bailliet and Kohorn 1996). The remaining 23 strains contained extragenic, nuclear mutations that described 6 loci. These mutant genes have been termed tips for thylakoid insertion proteins, and their characterization is described in this article.

MATERIALS AND METHODS

Strains, media and crosses: Strains containing cytochrome f+, (wt), cytochrome f with mutant signal sequences (cyt A15E, cyt V16D, cyt A15E V16D) and tip suppressed strains were generated as described in Smith and Kohorn (1994). All media were made according to Harris (1989). Cytomutant strains were propagated in the dark on plates containing acetate, 100 μg/ml spectinomycin, 100 μg/ml streptomycin, and 50 μg/ml ampicillin (tris-acetate-phosphate medium (TAP) sp stamp plates) and tip suppressed strains were kept in moderate light (15 μmol photons/m² sec) on acetate-minus antibiotic plates (high salt medium (HS) stamp plates). Crosses and tetrad analysis were performed as follows.

Diploid analysis: Diploid analysis was performed by mating a tip (pD1T1E mt+) and a vast excess of CC-48 (arg2 mt- TIP). Diploids mate as mt- and only diploids will be mt- and Arg-. (Harris 1989). Haplotype progeny of this mating did not occur as insufficient time was allowed for their development. Diploids become stable if maintained on nitrogen-rich media. Therefore, cells were plated at low density on TAP (no Arg) and after 3 days at ambient light, colonies (diploids) were picked and propagated on TAP plates.

Growth analysis by spot test: Strain growth was examined by spot test analysis to provide a clearer basis for comparison. Cells were propagated in patches on the appropriate medium for not longer than 7 days. 1 ml TAP cultures were inoculated from the patches and grown overnight with shaking in ambient light. Cell counts were performed on each culture. The cells were centrifuged for 3 min × 3000 g at room temperature and resuspended in HS at 2.5 × 10⁶ cells/ml. 10 μl, the equivalent of approximately 250 cells, was then spotted onto TAP and HS plates. The TAP plate was placed under medium light while the HS plate was grown under high light (200 μmol photons/m² sec). Both plates also contained spots of parental strains of the cross, CC-125, and cyt A15E as controls to compare growth between plates. After 10 days images were captured using the Eagle Eye (Stratagene, La Jolla, CA) still video system and NIH Image software.

Western blot analysis: Protein samples were prepared by harvesting cells from patches grown on TAP plates into 1 ml TE (10 mm Tris, pH 7.5, 1 mm EDTA). Cells were centrifuged at 5000 g for 5 min, washed with 1 ml TE and centrifuged again. The cell pellet was resuspended in 20 μl TE. One microliter was used to determine the protein concentration and the rest stored at −20º. Protein concentrations were determined by ODS, the equivalent of 5 μg chlorophyll (Porra et al. 1995) added to an equal volume of 2X sample dye (4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.2 m DTT, 0.5 m Tris, pH 6.8, and bromophenol blue) and boiled for 5 min.

Protein samples were separated by SDS-PAGE on 15% acrylamide gels and transferred to nitrocellulose (Schleicher & Schuell Inc., Keene, NH). The blot was blocked in 5% milk + TBS (10 mm Tris-HCl, pH 8.0, 150 mm NaCl, 1 mm EDTA, and 1% Triton X-100) overnight at 4º. It was then incubated with anti-cyt antisera (1:2000; Smith and Kohorn 1994) or anti-PC antisera (1:1000; gift of Sabeeha Merchant, University of California, Los Angeles) in 5% milk/ TBS for 3 hr and washed in TBS. The blot was incubated for 1 hr with HRP-conjugated anti-rabbit antisera (1:5000 in 5% milk/ TBS), and washed again with TBS. The secondary antibody was detected by chemiluminescence using the ECL reagents (Amer sham Life Sciences, Arlington Heights, III).

Pulse chase analysis: The mt- genotype of wt, cyt A15E, and a representative of each tip locus were labeled with H35SO4 (ICN Biomedicals Inc., Costa Mesa, CA) as described in Smith and Kohorn (1994). Briefly, cultures were grown to midlogar-
WT, wild type.

* Growth of strain under stringent photosynthetic conditions (high light, no acetate) as compared with CC-125 (progenitor strain): range between +++ (comparable growth) and (cells dead).

Chlamydomonas Genetics Center; Duke University, Durham, NC 27708.

isolate of this strain and selection for photosynthetic growth on acetate-minus media provided a collection of 27 strains containing suppressors of the cyt f A15E mutation (Smith and Kohorn 1994). No suppressors were identified in the absence of mutagen. These suppressor strains were each crossed to a mt- cyt f strain (CC-124) to determine the segregation pattern of the suppressor mutations. Cyt f is chloroplast encoded (petA gene) and is, therefore, usually inherited from the mt parent (Harris 1989). Suppression could result from a mutation in either a chloroplast or nuclear gene, the former resulting in all progeny of a tetrad being suppressed, segregating as chloroplastic alleles. Their characterization in either a chloroplast or nuclear gene, the former resulting in all progeny of a tetrad being suppressed, segregating as chloroplastic alleles. Their characterization and influence on the signal sequence has been described (Baillet and Kohorn 1996). Twenty-three of the twenty-seven suppressors showed Mendelian type (2:2) inheritance, identifying them as nuclear and, therefore, extragenic suppressors. Since these nuclear-encoded suppressors are candidates for mutations within the thylakoid translocation machinery components, they were named tips for thylakoid insertion protein.

Identification of six tip loci: To determine the number of tip loci, tetrad analysis of pairwise crosses between each of the tip strains was performed. The suppressors were isolated in mt+ strains and the mt- strains used in further crosses were identified from among the tip carrying progeny of the tip (petA A15E mt+) × CC-124 (petA+mt-) cross. Pairwise crosses between the 23 strains carrying nuclear suppressors were used to generate

### RESULTS

**Isolation of suppressors**: An Ala-to-Glu mutation at amino acid 15 (A15E) in the thylakoid signal sequence of cytochrome f (cytf) reduces the translocation of cytf, and under high light conditions renders Chlamydomonas reinhardtii unable to grow in the absence of acetate (on HS medium). EMS mutagenesis of 10⁸ cells of a mt+ isolate of this strain and selection for photosynthetic growth on acetate-minus media provided a collection of 27 strains containing suppressors of the cyt f A15E mutation (Smith and Kohorn 1994). No suppressors were identified in the absence of mutagen. These suppressor strains were each crossed to a mt- cyt f strain (CC-124) to determine the segregation pattern of the suppressor mutations. Cyt f is chloroplast encoded (petA gene) and is, therefore, usually inherited from the mt parent (Harris 1989). Suppression could result from a mutation in either a chloroplast or nuclear gene, the former resulting in all progeny of a tetrad being suppressed, segregating as chloroplastic alleles. Their characterization and influence on the signal sequence has been described (Baillet and Kohorn 1996). Twenty-three of the twenty-seven suppressors showed Mendelian type (2:2) inheritance, identifying them as nuclear and, therefore, extragenic suppressors. Since these nuclear-encoded suppressors are candidates for mutations within the thylakoid translocation machinery components, they were named tips for thylakoid insertion protein.

**Identification of six tip loci**: To determine the number of tip loci, tetrad analysis of pairwise crosses between each of the tip strains was performed. The suppressors were isolated in mt+ strains and the mt- strains used in further crosses were identified from among the tip carrying progeny of the tip (petA A15E mt+) × CC-124 (petA+mt-) cross. Pairwise crosses between the 23 strains carrying nuclear suppressors were used to generate

### TABLE 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth phenotype</th>
<th>Nuclear genotype</th>
<th>Chloroplast genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-124</td>
<td>+++</td>
<td>mt-agg1 nit1-137 nit2-137</td>
<td>petA+</td>
<td>CGC</td>
</tr>
<tr>
<td>CC-125</td>
<td>+++</td>
<td>mt-agg1 nit1-137 nit2-137</td>
<td>petA+</td>
<td>CGC</td>
</tr>
<tr>
<td>CC-48</td>
<td>+++</td>
<td>mt+ arg2</td>
<td>petA+</td>
<td>CGC</td>
</tr>
<tr>
<td>WT</td>
<td>+++</td>
<td>mt- agg1 nit1-137 nit2-137</td>
<td>petA+ spec' strep' petA A15E spec'</td>
<td>Smith and Kohorn (1994)</td>
</tr>
<tr>
<td>cytf A15E</td>
<td>–</td>
<td>mt+ agg1 nit1-137 nit2-137</td>
<td>strep' petA A15E spec'</td>
<td>Smith and Kohorn (1994)</td>
</tr>
<tr>
<td>cytf V16D</td>
<td>–</td>
<td>mt+ agg1 nit1-137 nit2-137</td>
<td>petA A15E spec' strep'</td>
<td>Smith and Kohorn (1994)</td>
</tr>
<tr>
<td>tip1-1</td>
<td>+++</td>
<td>mt- agg1 nit1-137 nit2-137 tip1-1</td>
<td>petA A15E spec' strep'</td>
<td>This study</td>
</tr>
<tr>
<td>tip2-1</td>
<td>++</td>
<td>mt- agg1 nit1-137 nit2-137 tip2-1</td>
<td>petA A15E spec' strep'</td>
<td>This study</td>
</tr>
<tr>
<td>tip3-1</td>
<td>++</td>
<td>mt- agg1 nit1-137 nit2-137 tip3-1</td>
<td>petA A15E spec' strep'</td>
<td>This study</td>
</tr>
<tr>
<td>tip4-1</td>
<td>++</td>
<td>mt- agg1 nit1-137 nit2-137 tip4-1</td>
<td>petA A15E spec' strep'</td>
<td>This study</td>
</tr>
<tr>
<td>tip5-1</td>
<td>+++</td>
<td>mt+ agg1 nit1-137 nit2-137 tip5-1</td>
<td>petA A15E spec' strep'</td>
<td>This study</td>
</tr>
<tr>
<td>tip6-1</td>
<td>+++</td>
<td>mt+ agg1 nit1-137 nit2-137 tip6-1</td>
<td>petA A15E spec' strep'</td>
<td>This study</td>
</tr>
</tbody>
</table>

WT, wild type.

* Growth of strain under stringent photosynthetic conditions (high light, no acetate) as compared with CC-125 (progenitor strain): range between +++ (comparable growth) and (cells dead).

Chlamydomonas reinhardtii
tetrads that were analyzed for photosynthetic growth on HS (no acetate) or heterotrophic growth on TAP (acetate medium). All progeny carried the cytF<sup>15E</sup> mutation and thus required the presence of a suppressor for photosynthetic growth on HS. Two alleles were considered linked if all of the four products from 11-29 tetrad grew on HS as this indicates that the tetrad were all parental ditypes (Harris 1989). Whenever a given cross indicated linkage further crosses of that strain with other suppressors were discontinued and more crosses between the two linked alleles in question were done to increase the sample size. As the eventual goal of this analysis is to clone the tip loci by library complementation the exact map location was not required although the tip1 and tip4 loci have been mapped to linkage group 12/13. When a suppressor in a new locus was identified, diploid analysis was performed to ascertain whether, as expected, the suppression was caused by a dominant mutation. Within the collection of 23 nuclear suppressors 6 groups of linked isolates were identified. Each group likely represents a single locus as we have been unable to find recombination within a group, but the sample size was insufficient to exclude the possibility of recombination between tightly linked mutations or to determine whether any of the identified loci mapped to the same linkage group. However, the isolates within a given locus may be different alleles as they show different sensitivity to sodium azide (P. Hertzog and B. Kohorn, unpublished data). To assure the phenotype of each tip-suppressed strain reflects the effect of a single mutation, a single isolate of each tip locus was back crossed two additional times and used in all further characterization (Table 1) and these are referred to as tip1-1, tip2-1, tip3-1, tip4-1, tip5-1, and tip6-1.

The tip suppressors were selected on the basis of restored autotrophic growth to the nonphotosynthetic cytF<sup>15E</sup> strain. Figure 1 shows the growth of each suppressor strain on HS and TAP. The cytF<sup>15E</sup> strain is impaired in growth on TAP, relative to wild type, and cannot grow at all photosynthetically on high light on HS. Each suppressor overcomes the growth defect to a different extent. Strains carrying cytF<sup>15E</sup> and either tip1-1, tip2-1, tip3-1, tip4-1, tip5-1, or tip6-1 exhibit growth on TAP that is much greater than the unsuppressed A15E parent. The tip mutations vary more significantly in their ability to restore photosynthetic growth on HS. Complete suppression of the nonphotosynthetic growth phenotype is afforded by tip1-1, tip5-1, and tip6-1 while tip2-1, tip3-1, and tip4-1 only restore partial growth on HS.

To determine if the tip mutations have any effect on growth in the absence of any cytF mutation, we generated strains carrying each tip allele in a wild-type chloroplast background by crossing the tip (cytF<sup>15E</sup> mt<sup>−</sup>) strains with a wild type mt<sup>+</sup> strain (CC-125) to produce F<sub>2</sub> progeny that necessarily all had wild-type chloroplasts. To characterize the phenotype of each tip allele in a wild-type background, spot tests were performed by plating each of the tetrad products onto TAP (permissive) and HS high light (selective) plates. Since the tip mutations are nuclear, half of the F<sub>2</sub> colonies of every tetrad should contain the suppressor. Thus if there were an effect by a tip mutation, two of the tetrad products should show an alteration in growth. All strains produced F<sub>2</sub> progeny (entire tetrads) with wild-type growth on HS and, thus, have no detectable effect in the absence of the chloroplast cytF<sup>15E</sup> mutation (K. Bernd and B. Kohorn, unpublished data).

**tip mutations restore cytF levels:** If suppression of the cytF<sup>15E</sup> signal sequence mutation was due to a compensating mutation within the translocation machinery, an increase in steady-state levels of mature cytF would be expected. Total cell extracts were, therefore, analyzed by Western blot using cytF antisera and the results are shown in Figure 2. Equal loading of protein was verified by immunological detection of plastocyanin (K. Bernd, unpublished data). The precursor form of cytF is never detected by Western blot in any strain, presumably due to limitations in the amount of protein loaded and precursor lability. Under high light conditions, the levels

---

**Figure 1:** Suppression of the cytF<sup>15E</sup> mutation varies between tip loci. Spot tests were performed using ~250 cells/spot and plating under permissive conditions (TAP, medium light) or stringent conditions (HS, photosynthetic growth, high light) for 10 days. Suppression is indicated by growth under stringent conditions (HS). WT, wild type strain; A15E, strain containing the cytF<sup>15E</sup> transit peptide mutation; tip/A15E, strain carrying a tip allele and a chloroplast cytF<sup>15E</sup> mutation.
of cyt f are undetectable in the cyt \textsuperscript{A15E} strain (Figure 2, lane A15E). The suppression provided by tip 1-1, 2-1, and 4-1 allows accumulation of cyt f\textsuperscript{A15E} to approximately 50\% of wild type, while tip 3-1, 5-1, and 6-1 have steady-state levels comparable to those seen in a cyt f\textsuperscript{A15E} strain. Thus photosynthetic growth in the suppressed strains indeed is accompanied by increased cyt f levels. The steady-state levels of cyt f as detected by Western analysis, however, do not correlate with the degree of photosynthetic growth (Figure 1).

**tip mutations effect translocation:** Wild type cyt f is processed from a 35-kD stromal precursor to the mature 32-kD thylakoid membrane form by a luminal peptidase (Gray 1992). The A15E mutation causes the accumulation of precursor cyt f in the stroma and on the surface of the thylakoid while affecting neither mRNA nor translation levels (Smith and Kohorn 1994). To determine if the increased steady-state levels of cyt f in the suppressed strains resulted from a change in cyt f translocation, the tip strains were subjected to pulse-chase analysis.

Cells were grown in media containing reduced sulfur, labeled with H\textsubscript{2}\textsuperscript{35}SO\textsubscript{4}, and aliquots were removed at 5, 15 and 30 minutes during a chase with excess cold sulfate. Cyt f was immunoprecipitated from the aliquots and visualized by SDS-PAGE and autoradiography, and the results are shown in Figure 3. As expected, mature cyt f, but never precursor cyt f is detected in the wild type (WT), whereas only low levels of precursor are seen in the cyt f\textsuperscript{A15E} strain. The amount of mature cyt f seen in the tip strains varies between that seen in tip3-1, where processing is most similar to wild type, to that seen in tip4-1, where, reproducibly, some cyt f is processed to the mature form but much remains as precursor even after a 30-min chase. No strain exhibited a dramatic increase in the net rate of synthesis of cyt f over and above that of wild type, indicating that protein synthesis is likely unaffected. These data suggest that the tip suppressors act by restoring the rate of translocation of mutant cyt f precursor.

**Allele specificity:** Suppressor mutations can arise through a specific alteration in one protein that directly compensates for a mutation in another protein. This type of suppression may be expected to be allele specific. Alternatively, suppression can arise from a less specific interaction between the mutant protein and the suppressor. This type of suppression should not be allele specific and should be capable of restoring the translocation of other signal sequence mutations. Mutation of Val-to-Asp at residue 16 (V16D) or deletion of the hydrophobic core region (hc\textalpha) of the cyt f transit peptide also inhibit cyt f translocation and cause the strain to be nonphotosynthetic (Smith and Kohorn 1994). The six tip mt\textsuperscript{+} strains were mated with strains having a wild-type nuclear genome but carrying either the V16D or hc\textalpha mutation in the chloroplast cyt f. Since the cyt f mutation is only inherited from the mt\textsuperscript{+} strain all progeny should inherit the cyt f\textsuperscript{V16D} or cyt f\textsuperscript{hc\textalpha} mutation and only half should inherit a nuclear tip allele. As expected, the F\textsubscript{1} of all crosses were able to grow on TAP (K. Bernd, unpublished data). The F\textsubscript{1} were then screened for photoautotrophic growth on HS under high light. Crosses with all tip alleles to cyt f\textsuperscript{hc\textalpha} produced tetrads with a segregation of 2 live: 2 dead on HS. Thus all of the tip mutations are capable of suppressing the cyt f\textsuperscript{hc\textalpha} mutation. Representatives of the progeny that grew on HS are shown in Figure 4. In contrast, only tip5-1 and tip2-1 and not the other tip alleles can suppress cyt f\textsuperscript{V16D}, although tip2-1 is very weak (Figure 4). The data indicate that while the tip mutations cannot accommodate all mutant transit peptides, they are nonallele specific.

**Dominance:** The tip mutations permit growth of strains with deficient signal sequences, but also still allow the translocation of other wild-type thylakoid proteins. The tip alleles would therefore be expected to be dominant and to allow a diploid strain heterologous for the suppressor to grow photosynthetically. Since Chlamydomonas is a vegetative haploid, to test for dominance we generated diploid strains carrying one tip allele and a wild-type TIP loci in addition to cyt f\textsuperscript{A15E} (see materials and methods; Harris 1989). All of the diploids grew photosynthetically (HS, high light) confirming that all
Figure 4.—tip mutants are nonallele-specific suppressors. Spot tests plating ~250 cells under stringent growth conditions (HS, high light) were performed on tip strains containing the cyt A15E, hcΔ or V16D mutations (indicated above each column). Suppression of the cyt mutation results in growth of cells within the spot.

Interaction between tips: It is possible that cyt translocation is mediated by a protein complex encoded in part by the Tip loci. If the proteins encoded by the Tip loci interact with each other, then combinations of tip mutations within one strain might be expected to show different levels of suppression than are seen in strains with either tip mutation alone. To test this prediction, strains containing all possible pairwise combinations of tips in a cyt A15E background were constructed by crossing a tip petA A15E mt− strain to a tip petAA15E mt+ strain. The crosses were performed in a cyt A15E background because the tip suppressors have no detectable phenotype alone. Progeny in the F1 that contained two tip alleles were identified applying the following reasoning; in a cross between any two unlinked genes three types of tetrads arise: parental ditypes (PD; all have parental genotypes), nonparental ditypes (NPD; all have recombinant genotypes) and tetratypes (T; one of each parental genotype and one of each recombinant genotype). Therefore, in crosses concerning two unlinked tip mutations in a cyt A15E background, if there was no effect from a combination of two, then growth on HS would show tetrads with the following growth phenotypes PD 4:0, NPD 2:2 and T 3:1 (growth: no growth); the presence of one or two tip suppressors in a spore allows its growth. However, if there was a detrimental combinato-

Figure 5.—Growth of strains containing two tip mutations indicates genetic interactions between tips. Spot tests of strains containing pair-wise combinations of the tips in a cyt A15E background. Spots contain ~250 cells grown under stringent conditions (HS, high light) for 10 days.

six tip alleles tested are dominant (K. Bernd, unpublished data).

DISCUSSION

To identify components that mediate the translocation of proteins into and across the thylakoid membrane we selected 23 nuclear suppressor strains of Chlamydo-
monas. These strains describe 6 Tip loci that can be mutated to suppress the inhibitory effect of a signal sequence mutation of cytf. Thus at least 6 nuclear-encoded components are involved in the translocation of cytf into the thylakoid membrane. Since biochemical (Hig et al. 1997) and genetic evidence (Smith and Kohorn 1994; Voelker and Barkan 1995) indicates that cytf is translocated by components shared with nuclear-encoded thylakoid membrane proteins, it is possible that some of the encoded Tip products mediate translocation of a number of different thylakoid proteins, and have the potential to describe much of the functional apparatus.

We chose to analyze one mutant allele of each Tip locus in more detail. Pulse-chase analysis indicated that the tip mutations act by increasing the rate of maturation of mutant precursor cytf, although each tip allele does this to varying degrees. Suppression does not appear to be due to increased protein synthesis and the tip suppressors do not significantly alter the fitness of cells in the absence of the A15E mutation, even under acetate-depleted conditions that require thylakoid function. This is consistent with the idea that the tip suppressors are directly involved in protein translocation and are not general regulators of chloroplast gene expression. The lack of an effect on other wild-type proteins also indicates that the tip suppressors are mutations that make the translocation machinery generally more permissive. Thus it is also not surprising that each of the tip mutations is dominant, as determined by diploid analysis (K. Bernd, unpublished data).

The tip mutations were selected for their ability to suppress the petA A15E mutation but crosses with Chlamydomonas strains carrying other cytf signal sequence mutations showed that the tips can also suppress these, albeit to differing degrees. Thus the tips are not allele specific. The original expectation was that a given suppressor would compensate for a specific signal mutation. However, as the tip mutations can suppress even a deletion of the otherwise essential hydrophobic core of the signal sequence, the data suggest that the suppressors render the thylakoid more permissive to translocation so as to accommodate a variety of types of signals, including wild type, less than ideal signals, or no signal at all. This same observation is noted for similar selection schemes in bacteria (Emr et al. 1981). If the tip suppressors are allowing promiscuity of translocation one might expect to detect unusually high levels of inappropriate proteins in the thylakoid. Pulse-labeling of whole cells or of chloroplasts isolated from the tip strains was unable to detect any unusual protein profiles so some degree of selectivity remains (K. Bernd and B. D. Kohorn, unpublished results). Bacterial alleles that are permissive for mutant signal sequences also do not accumulate inappropriate proteins (Emr et al. 1981). The tip suppressors are being cloned by ordered library transformation of the cytf A15E strain and hopefully characterization of these loci will resolve some of these issues.

The tip mutations suppress cytf A15E to different extents, where tip1-1 and tip5-1 are the most effective in restoring photosynthetic growth. However, all of the tip strains do not show a correlation between their growth phenotype and cytf levels detected either by pulse chase or Western blotting. Colonies of tip1-1 exhibit impaired cytf A15E processing but wild-type growth while those of tip3-1 show decreased growth yet cytf A15E processing approaches wild-type rates (Figure 1 vs. Figure 3). This may indicate that both the rate of maturation and the absolute levels of cytf are important in determining phototrophic growth, but their relationship is not understood. It is also possible that the tip mutations affect the maturation of other proteins in ways not detected by pulse labeling.

Significant accumulation of mature cytf is seen by both pulse-chase and Western blot analysis in tip2-1, tip3-1, and tip5-1. Although by pulse-chase analysis tip2 appears to accumulate mature cytf to similar extents as tip5, it is unclear why their steady-state levels seen on Western blots differ. Tip1-1 and tip4-1, while showing increased accumulated cytf by Western blot, do not have a significant increase in levels of mature cytf in pulse-chase analysis. This discrepancy is not understood, but may indicate that these two suppressors affect cytf maturation at steps not directly involved in translocation per se. In tip1 and tip4, increased expression could lead to higher levels of mature cytf A15E protein, but such dramatic expression changes are not seen. Suppressor mutations that alter translation efficiency would also be expected to show genetic interaction with all of the other tip suppressors, but only tip4 and tip5 were seen to interact genetically. Even if tip1-1 and tip4-1 lie in proteins accessory to the translocation apparatus their identification will provide a context for the other tips.

Of the six tip alleles tested, only tip5-1, and to a lesser extent tip2-1, can suppress the cytf translocation block caused by the cytf V16D mutation under highly stringent conditions. The variations in suppression may reflect the stage at which the Tips interact with the targeted protein. The cytf V16D mutation inhibits cytf from binding the thylakoid membrane, while the cytf A15E mutation appears to block cytf at the surface of the thylakoid and during its transfer (Smith and Kohorn 1994). Since tip5-1 is able to suppress cytf V16D, Tip5 protein (Tip5p) could act in an initial step of transit peptide recognition and commitment to translocation. Similarly, Tip2p may also be involved at this early stage, but be more peripheral in action as tip2-1 only weakly suppresses cytf V16D. However, additional tip2 alleles may have stronger effects. Double mutant analysis fails to provide evidence of a tip2-1 tip5-1 interaction (Figure 5). Since the V16D mutation appears to affect the initial recognition and entry of cytf into the translocation pathway, tip1-1, 3-1,
4-1 and 6-1 may not suppress this mutation because they act at steps that this mutant precursor could not reach.

All six tip mutations suppress a deletion of the cyt hydrophobic core (cyt F invaluable to the addition of a single charged residue within this region (cyt V16D). The site of blockage in cyt F+nucleus translation is not known as the precursor is extremely labile. It is clear that other cryptic hydrophobic regions of the signal sequence can serve to provide a functional signal if there is selective pressure (Baillet and Kohorn 1996). The tip suppressors may recognize such a cryptic domain in an otherwise normally folded cyt transit peptide more easily than overcoming the addition of charge and possible folding problems caused by the cyt V16D mutation in this region.

Genetic interaction between tip alleles is also suggested by the data. Strains containing either tip4-1 or tip5-1 demonstrate more photosynthetic growth than their nonsuppressed cyt A15E strain (Figure 1). Strains containing both tip4-1 and tip5-1, however, do not grow under stringent conditions. This may indicate that Tip4p and Tip5p directly interact: the mutation that allows each to suppress transit peptide mutations may also prevent it from interacting productively with the other tip suppressor. That the tip4-1 or tip5-1 mutation has no effect alone on cell growth argues that their combinatorial inhibitory effect is not simply additive.

The biochemical analysis of protein translocation in isolated organelles (Cline and Henry 1996) suggests multiple requirements for the translocation of different precursors, and this is believed to be representative of multiple translocation pathways in the thylakoid. The tip mutations may lie in at least one of these pathways. Suppression of a mutation in the cyt signal might also be achieved by the acceptance of that mutant signal by an otherwise distinct path that normally accommodates other precursors. Thus if there are multiple paths, the tips have the potential to describe these in addition to the mechanism mediating cyt translocation. It will be of interest to see if the various tip suppressors have an influence on inhibitory mutations in a variety of different signal sequences as this may determine whether the numerous pathways are directed by one or multiple machinery.

We thank Phillip Hartzog for assisting with initial crosses of tip suppressors and noting the azide sensitivity of some strains Elizabeth H Harris and Michele Perr et for critical reading of the manuscript and Zheng Hui He for helpful discussions. The anti-plastocyanin antiserum was a generous gift of Dr. Sabeeha Merchant (UCLA). This research was supported by the United States Department of Agriculture (Grant 9502733) to B.D.K. and the National Institutes of Health, National Research Service Award (1 F32 GM18892-01) to K.K.B.

LITERATURE CITED


Yuan, J., and K. Cline, 1994 Plastocyanin and the 33-kDa subunit of the oxygen-evolving complex are transported into thylakoids with similar requirements as predicted from pathway specificity. J. Biol. Chem. 269: 18463–18467.

Communicating editor: J. Chory