TCA1, a Single Nuclear-Encoded Translational Activator Specific for *petA* mRNA in *Chlamydomonas reinhardtii* Chloroplast

K. Wostrikoff, Y. Choquet, F.-A. Wollman and J. Girard-Bascou

UPR/CNRS 1261, Institut de Biologie Physico-Chimique, 75005 Paris, France

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ABSTRACT

We isolated seven allelic nuclear mutants of *Chlamydomonas reinhardtii* specifically blocked in the translation of cytochrome *f*, a major chloroplast-encoded subunit of the photosynthetic electron transport chain encoded by the *petA* gene. We recovered one chloroplast suppressor in which the coding region of *petA* was now expressed under the control of a duplicated 5′ untranslated region from another open reading frame of presently unknown function. Since we also recovered 14 nuclear intragenic suppressors, we ended up with 21 alleles of a single nuclear gene we called *TCA1* for *t*ranslation of *c*ytochrome b_6f complex *petA* mRNA. The high number of *TCA1* alleles, together with the absence of genetic evidence for other nuclear loci controlling translation of the chloroplast *petA* gene, strongly suggests that TCA1 is the only *trans*-acting factor. We studied the assembly-dependent regulation of cytochrome *f* translation—known as the CES process—in *TCA1*-mutated contexts. In the presence of a leaky *tca1* allele, we observed that the regulation of cytochrome *f* translation was now exerted within the limits of the restricted translational activation conferred by the altered version of TCA1 as predicted if TCA1 was the ternary effector involved in the CES process.

THE well-developed tools for genetic analysis in *Chlamydomonas reinhardtii* offer a unique opportunity to study the regulation of chloroplast gene expression *in vivo* and more specifically the control exerted by the nucleus on post-transcriptional steps such as mRNA maturation, stabilization, and translation. As discussed in Wollman *et al.* (1999), translation appears as the main regulatory step in the expression of organellar genes. This is well documented both in yeast mitochondria (reviewed in Fox 1996) and in chloroplasts from higher plants (Gamble and Mullet 1989; Barkan *et al.* 1994; Kim *et al.* 1994 ; Fisk *et al.* 1999; Mccormac and Barkan 1999) or green algae (reviewed in Zerges 2000). In *C. reinhardtii*, a number of nuclear mutants are specifically altered in the translation of a single organellar gene. Nuclear-encoded factors acting at the translational step were identified for *atpA* (Drapier *et al.* 1992), *psaB* (Stampacchia *et al.* 1997), *psbA* (Girard-Bascou *et al.* 1992; Yohn *et al.* 1998), *psbC* (Rochaix *et al.* 1989; Zerges and Rochaix 1994; Zerges *et al.* 1997), and *psbD* (Kuchka *et al.* 1988). While the nuclear mutations affecting *psbD* translation may act at the level of elongation or stabilization of the nascent product (Wu and Kuchka 1995; Rattanachaikunsopon *et al.* 1999), all other nuclear factors are specific activators of translation acting on the 5′ untranslated region (UTR) of their target mRNA. In most cases we still do not know whether these factors are merely constitutive of chloroplast gene expression or have a genuine regulatory function.

In *C. reinhardtii*, the rate of translation of several chloroplast-encoded polypeptides also depends on the presence of those polypeptides with which they ultimately assemble in an oligomeric protein (for reviews see Wollman *et al.* 1999; Choquet and Vallon 2000). This process was termed "*c*ontrol by *e*pistasy of *s*ynthesis" (CES) to account for the experimental observation that the synthesis of a CES subunit is markedly reduced in the absence of its assembly partners, viewed as "dominant" subunits from the same protein complex. To date, cytochrome *f*, a subunit of the cytochrome b_6f complex encoded by the chloroplast *petA* gene, is the best-characterized CES subunit (Kuras and Wollman 1994; Choquet *et al.* 1998). At the molecular level, we have shown that the assembly-mediated control of cytochrome *f* synthesis is an autoregulation of translation initiation (Choquet *et al.* 1998). Still, the nature of the interaction between a regulatory motif in unassembled cytochrome *f* and the 5′ UTR of the *petA* transcript is not known. Cytochrome *f* has no reported RNA-binding activity and displays no typical RNA-binding motif. Thus, the interaction is likely to be indirect. It would rely on the competitive binding of a translation activator to unassembled cytochrome *f* and to the *petA*-5′ UTR. In this model, repression of cytochrome *f* synthesis, as observed in the absence of subunit IV (Kuras and Wollman 1994), should result from a lack of translational activation.

The aim of this study is to dissect genetically the specific nuclear control of *petA* translation and to explore

Corresponding author: J. Girard-Bascou, UPR/CNRS 1261, Institut de Biologie Physico-Chimique, 13 rue P. et M. Curie, 75005 Paris, France. E-mail: girard@ibpc.fr

the links between the CES process and the translation of *petA* mRNA mediated by *trans*-acting factors of nuclear origin.

MATERIALS AND METHODS

Media, culture conditions, and strains: Wild-type and mutant strains were grown on Tris-acetate-phosphate (TAP) medium, pH 7.2, at 25° under dim light (5–6 μE m^{-2} sec^{-1}), unless otherwise indicated. To assay phototrophic growth, cells were streaked on minimal medium plates and allowed to grow under an illumination of 80 μE m^{-2} sec^{-1} for 10 days. Antibiotic resistance tests were performed as described in Choquet *et al.* (1998) on TAP plates supplemented with various concentrations of spectinomycin and streptomycin as indicated. Antibiotic concentrations were corrected for the percentage of impurity of the batches.

For genetic crosses and chloroplast transformation we used wild-type strains of *C. reinhardtii* from our laboratory that are derived from the original 137c strains. The nuclear mutant strains used in this study were *mcd1-F16, mt−* (Drager *et al.* 1998) and *mca1-MΦ11* (Girard-Bascou *et al.* 1995; Gumpel *et al.* 1995). The chloroplast mutants were the deletion strains Δ*petD, mt+* and Δ*petA, mt+* (Kuras and Wollman 1994) and the *mt+* chloroplast transformant *KF303Q304St*, where the first Lysine (K_{303}) of the stromal extension of cytochrome *f* is substituted by a Glutamine and immediately followed by a stop codon that truncates the protein by its last 14 residues.

Genetic analysis: As a convention, all crosses are indicated with the *mt+* parent first, *i.e.*, the strain whose chloroplast genome is transmitted to the whole progeny. For gametogenesis, the cells were grown for 3–4 days on TAP plates containing one-tenth the usual amount of nitrogen. Mating, germination, and tetrad analysis were performed according to Harris (1989). Germination of zygotes was controlled to be >75% unless otherwise indicated. Tetrad progeny were tested for a 2:2 segregation of mating types. For some experiments we pooled the meiotic products from all tetrads even if some were incomplete. Reversion tests and recombination analysis were performed as described in Kuras *et al.* (1997), while complementation analysis was done according to Goldschmidt-Clermont *et al.* (1990).

Isolation of mutant strains: Eight mutants deficient in cytochrome *f* synthesis are described in this work. Six of these, *tca1-1*, *tca1-3*, *tca1-4*, *tca1-5*, *tca1-6*, and *mca1-792*, were identified among a population of UV-mutagenized CC125 (*mt+*) cells (Girard-Bascou *et al.* 1995; Xie *et al.* 1998). Two other mutant strains, *tca1-2* and *tca1-7*, were screened out of a population of FdUrd-mutagenized wild-type cells from our laboratory. Mutagenesis using 5-fluorodeoxyuridine (FdUrd) was achieved at a concentration of 1 mm (Wurtz *et al.* 1979). UV irradiation was performed as in Li *et al.* (1996), followed by an enrichment step in the presence of metronidazole according to Bennoun and Delepelaire (1982). Cytochrome b_6f mutants were screened for their fluorescence yield as described in Zito *et al.* (1997), using a video-imaging system built in-house (Bennoun and Beal 1997).

Isolation of diploid strains: Vegetative diploids were isolated according to published protocols (Harris 1989), using complementation between *arg2* and *arg7* mutations.

Isolation of revertant strains: Revertants were isolated from *tca1, mt+* strains using various mutagenic agents. UV mutagenesis was conducted as described in Girard *et al.* (1980). EMS mutagenesis was performed with exponentially growing cells on pretreated cells that were grown for 4 days on TAP plates containing 0.1 mm FdUrd or on gametes. Cells were treated with 2% EMS for 30 min, washed three times, allowed to recover for 2 days in TAP liquid medium, and then selected in liquid minimal medium. Viability after mutagenesis, from 25 to 80% depending on batches, was estimated by counting the cells prior to and after treatment. FdUrd mutagenesis was performed on TAP plates containing 1 mm FdUrd (Wurtz *et al.* 1979). Typically, reversion became detectable after 3–5 weeks of culture in minimal medium under high light illumination (80 μE m^{-2} sec^{-1}). Cells were subcloned and only one revertant clone per mutagenesis flask was retained.

Transformation experiments: Cells were transformed by tungsten particle bombardment as previously described (Kuras and Wollman 1994) with a helium particle gun built in-house by D. Béal, according to Takahashi *et al.* (1991). Phototrophic transformants were selected on minimum medium under high light (80 μE m^{-2} sec^{-1}). Transformants containing the *aadA* cassette were selected on TAP-spectinomycin-containing plates (60 μg ml^{-1}) and subcloned on the same medium under dim light (5–6 μE m^{-2} sec^{-1}) until they reached homoplasmy, as determined by DNA filter hybridization. At least three independent transformants were analyzed for each construct.

Nucleic acid manipulation: Plasmids pWQ encompassing the wild-type *petD* gene, Δ*petB* containing a deletion of cytochrome b_6-coding sequences (Kuras and Wollman 1994), and pFKR12 (Choquet *et al.* 1998) were described previously. For Northern analysis, total RNAs were extracted from whole cells and analyzed as described in Drapier *et al.* (1998), using *petA* and *petD* DNA probes described in Buschlen *et al.* (1991). The *atpB* probe is the 2.9-kb *Eco*RI-*Kpn*I fragment of the Ba5 chloroplast DNA fragment (Drapier *et al.* 1992). Probe *aadA* was obtained by a *Nco*I-*Hin*dIII digestion of plasmid pUC-atpX-AAD (Goldschmidt-Clermont 1991). For Southern blots, the 2.3-kb *Hin*dIII probe was prepared from a *Hin*dIII digestion of the piAH1.9 plasmid (Kuras and Wollman 1994). The *petA*-5′ UTR probe was prepared by PCR, using plasmid pWF (Kuras and Wollman 1994) as a template and oligonucleotides FT7Sac and PETAAUG as primers (probe B). Probe D was obtained by a *Hin*dIII-*Hin*fI digestion of a PCR product obtained using oligonucleotides R1cod3 and PETArevA as primers and chloroplast DNA from the Su_C, *tca1-2* strain as a template.

Chloroplast DNA manipulation: Purified chloroplast DNA was isolated as described in Choquet *et al.* (1992). For Southern blots, digestion products were separated on 0.7% TBE-agarose gels and transferred onto nylon membranes by capillarity. The 2-kb *Hin*dIII fragment of the Su_C, *tca1-2* strain was recovered from a *Hin*dIII bank of the Su_C, *tca1-2* chloroplast DNA cloned in the pUN121 vector (Nilsson *et al.* 1983), probed with the 2.3-kb *Hin*dIII wild-type probe (see Figure 6A). It was sequenced using primers designed in the pUN121 vector, pUN121codH and pUN121revH. The remaining reorganized DNA was amplified by PCR, using chloroplast DNA of the Su_C, *tca1-2* strain as a template and oligonucleotides PETArevA and R1cod3 as primers.

Oligonucleotides used for sequencing and/or PCR:
pUN121codH: GGTTGAAGGTAATTCCATGACCG
pUN121revH: GCTCAACAGCCTGCTCAGGGTCAA
R1cod1: CGTTACAGGCATGAGCTAGTA
R1cod2: GAATTTTAGTGGCAGTTGCCTCCT
R1cod3: GTTACTACTTCCTCGAGACAGAACC
R1rev1: CTGGTTAGAGTCTATCGAGCA
FT7Sac: CGCGAGCTCAGATATAATATATGTTGAGAAG AAAAAAAATAAAATTTAAATAGT
PETArevA: ACAGCTTGTGGTACTTCGATTTCAACTGCT
PETAAUG: GCGGATCCATGGACATAATTTTATTAA TCTTAAAACG

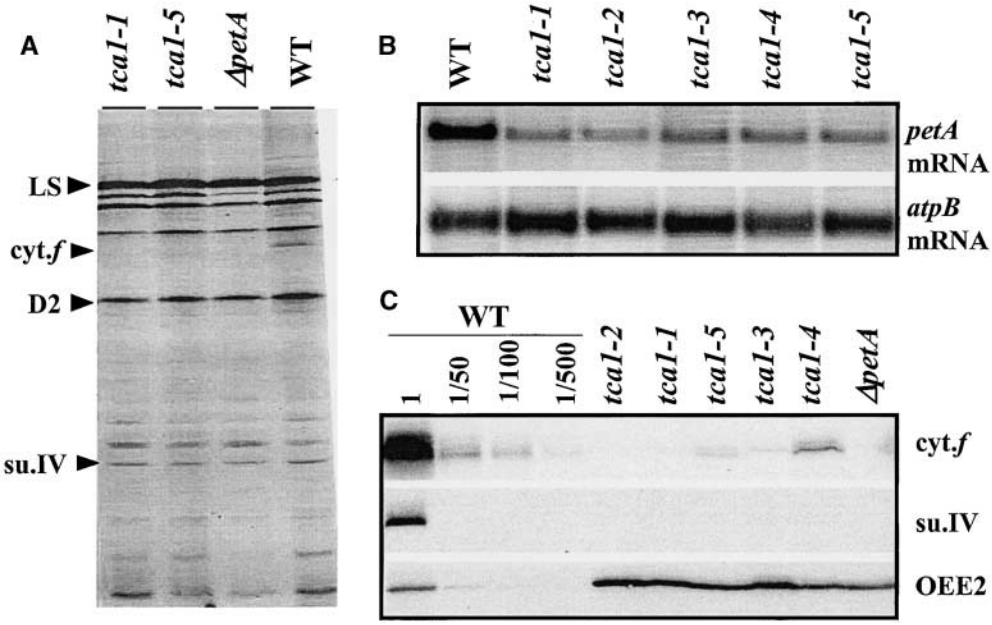

FIGURE 1.—*tca1* mutants are deficient in cytochrome *f* translation. (A) Chloroplast translates in the mutant strains *tca1-1* and *tca1-5* compared to those of the wild-type strain and of the deletion strain Δ*petA*. Other *tca1* mutants are indistinguishable from those two. (B) Accumulation of *petA* mRNA in wild-type and *tca1* mutant strains. *atpB* mRNA accumulation is presented as a loading control. (C) Accumulation of cytochrome *f* and subunit IV polypeptides from cytochrome b_6f complex in *tca1* mutants and in wild type, detected using specific antibodies. OEE2 accumulation is presented as a loading control. *tca1-6* and *-7* had a similar phenotype as the *tca1-1* and *-2* representative stringent mutant strains.

Protein isolation, separation, and analysis: Pulse-labeling experiments, protein isolation, separation, and analysis were carried out on cells grown to a density of 2×10^6 cells ml^{-1}, according to KURAS and WOLLMAN (1994).

RESULTS

Identification of nuclear mutants deficient in cytochrome *f* synthesis: More than 80 mutants lacking cytochrome b_6f activity have been generated by either UV mutagenesis (XIE *et al.* 1998) or FdUrd treatment. They were identified as being deficient both in immunoreactive forms of cytochrome *f* and in cytochrome b_6f activity on the basis of their typical fluorescence induction kinetics (Cyt b_6f^- phenotype; BENNOUN and DELEPELAIRE 1982; ZITO *et al.* 1997). Twelve mutants were specifically affected in cytochrome *f* synthesis as illustrated in Figure 1A by *tca1-1* and *tca1-5* strains that showed no detectable cytochrome *f* in 5-min pulse-labeling experiments with [^{14}C]acetate. Seven mutants, referred to as class I mutants and named *tca1-1* to *-7*, still displayed *petA* mRNA accumulation, albeit at reduced levels, from 15 to 30% of the wild-type amount (Figure 1B). *mca1-MΦ11*, a mutant strain previously described (GUMPEL *et al.* 1995), was totally deficient in *petA* mRNA accumulation as were four other mutants that we refer to as class II mutants. Cytochrome *f* accumulation was assayed in class I mutants by immunoblotting experiments (Figure 1C). Strains *tca1-3*, *-4*, and *-5* accumulated 0.1, 1.6, and 0.2% of wild-type accumulation of cytochrome *f*, respectively [whereas strains *tca1-1*, *-2*, *-6*, and *-7* showed no or quite undetectable amounts of cytochrome *f* (see *tca1-1* and *tca1-2* in Figure 1C)]. The other subunits of the cytochrome b_6f complex, such as subunit IV (Figure 1C) or cytochrome b_6 (not shown), accumulated only in trace amounts in the *tca1* mutants, in agreement with previous reports (LEMAIRE *et al.* 1986; KURAS and WOLLMAN 1994).

Genetic analysis of *tca1* mutants: The seven class I mutants (Cyt b_6f^- phenotype) affected in cytochrome *f* synthesis were backcrossed with the wild-type strain (Cyt b_6f^+ phenotype) to determine the inheritance of the mutant phenotype. All tetrads showed a 2:2 segregation for the Cyt b_6f^-:Cyt b_6f^+ phenotypes, indicating that the Cyt b_6f^- phenotype was due to a single nuclear mutation. We tested the recessivity of the *tca1-1* mutation by generating heterozygous vegetative diploids that exhibited the same fluorescence induction kinetics and phototrophic growth as did wild-type vegetative diploid cells. The seven *tca1* mutants were analyzed in recombination and complementation tests (Table 1). We used a rapid recombination test to detect mutations at the same locus. The progeny of individual zygotes derived from pairwise crosses between all mutants, except *tca1-5,* were tested for phototrophy on minimal medium. None gave rise to a recombinant progeny capable of phototrophic growth. Thus, all genetic distances were below 2.4 cM, indicative of a tight linkage. By contrast, no linkage was detected in crosses with the five class II mutant strains since phototrophic recombinants were recovered at high frequency (see Table 1, where *mca1-792* is taken as representative of class II mutants). Percentages of zygotes giving rise to wild-type progeny, which corresponds to the frequency of tetratype and nonparental ditype tetrads, were high indicating that the mutations probably affect two independent genes. In *C. reinhardtii*, complementation tests between photosynthetic mutants can be performed by testing the fluorescence induction kinetics of layers of young zygotes obtained from pairwise crosses (GOLDSCHMIDT-CLER-

TABLE 1

Genetic analysis of *tca1* mutants

Gene	Mutant	*1−1* UV	*1−2* FdUrd	*1−3* UV	*1−4* UV	*1−5* UV	*1−6* UV	*1−7* FdUrd	*792* UV
TCA1	*tca1-1*	10^{-9}	−	−	−	−	−	−	+
	tca1-2	0/27 (<1.8 cM)	$\leq 10^{-9}$	−	−	ND	−	−	+
	tca1-3	0/30 (<1.6 cM)	0/48 (<1.0 cM)	$\leq 10^{-9}$	−	−	−	−	+
	tca1-4	0/21 (<2.4 cM)	0/30 (<1.6 cM)	0/34 (<1.5 cM)	8×10^{-8}	ND	−	−	+
	tca1-5	ND	ND	ND	ND	3×10^{-7}	−	−	+
	tca1-6	ND	0/138 (<0.4 cM)	0/24 (<2.1 cM)	0/31 (<1.6 cM)	0/33 (<1.5 cM)	$\leq 10^{-9}$	−	+
	tca1-7	ND	0/24 (<2.1 cM)	ND	0/23 (<2.2 cM)	ND	0/27 (<1.8 cM)	10^{-8}	+
MCA1 (%)	*mca1-792*	31/50 (62)	15/28 (53)	21/24 (87)	17/31 (55)	ND	23/28 (82)	30/40 (75)	9×10^{-7}

Results of the complementation analysis are shown above the diagonal. The zygotes displayed either Cyt b_6f^- phenotype (indicated as "−") or wild-type phenotype (indicated as "+"). Results of the recombination tests are shown below the diagonal. The scores represent $a1/(a2 \times b1/b2)$, where $a1$ is the number of zygotes that germinated and gave rise to colonies on minimal medium, $a2$ is the number of zygotes transferred to minimal medium, $b1$ is the number of zygotes that gave rise to colonies on TAP medium, and $b2$ is the number of zygotes transferred to TAP medium. When we did not recover any zygotes giving rise to phototrophic progeny, we indicated in parentheses a maximum genetic distance. The results of the same tests performed between *tca1* mutants and the representative class II *mca1-792* mutant strain are shown as a control. Percentages of zygotes giving rise to phototrophic recombinant progeny are indicated in parentheses. Frequencies of spontaneous reversion of mutations are shown in diagonal. ND, not determined.

MONT *et al.* 1990). In crosses between the seven *tca1* mutants with the five class II mutants, young zygotes showed wild-type fluorescence induction kinetics, thus demonstrating altogether genetic complementation between the two mutant classes and recessivity of all mutations. In contrast, pairwise crosses between all class I mutants yielded young zygotes that had retained fluorescence induction kinetics of cytochrome b_6f^- mutants, indicating an absence of complementation. Thus, the seven class I nuclear mutations corresponded to recessive alleles of a single nuclear gene, which we called *TCA1* (**t**ranslation of **c**ytochrome b_6f *petA* mRNA).

The 5′ UTR of *petA* mRNA is the target of TCA1: We investigated the putative role of the *petA*-5′ UTR as a target for the translational control mediated by the *TCA1* nuclear gene product. To this end, we analyzed the expression of two chloroplast chimeric genes, *AFFF* and *FKR12*, in *TCA1* or *tca1* nuclear contexts. *AFFF* is a chimeric gene where the regular 5′ UTR of the *petA* gene has been substituted by the *atpA*-5′ UTR that preserves the phototrophic property of the *AFFF* strain (CHOQUET *et al.* 1998; Figure 2A). *FKR12* is a reporter gene driven by the *petA*-5′ UTR that confers resistance to spectinomycin and streptomycin in a wild-type nuclear context (CHOQUET *et al.* 1998; Figure 3A). We crossed mating-type plus (*mt*+) strains bearing *FKR12* or *AFFF* genes with a mating-type minus (*mt*−) *tca1-1* mutant strain. In the two crosses, the chloroplast chimeric genes should be mainly transmitted uniparentally in tetrads while the nuclear mutation *tca1* should be transmitted to only one-half of the progeny.

In *AFFF, mt+ × tca1-1, mt−* crosses, the whole progeny from 12 tetrads was phototrophic. As shown for one representative tetrad in Figure 2B, the rate of cytochrome *f* synthesis was similar in the four daughter cells. Accordingly, cytochrome *f* accumulated to the same extent in the four members of the tetrad (Figure 2B, bottom). No changes in *petA* mRNA levels were observed among the tetrad progeny (data not shown). Thus, translation of cytochrome *f* driven by the *atpA*-5′ UTR is no longer dependent upon the wild-type allele of the *TCA1* gene. This observation demonstrates that the mRNA target for TCA1 includes elements from the *petA*-5′ UTR.

In *FKR12, mt+ × tca1-1, mt−* crosses, the two *tca1* meiotic products were detected by their Cyt b_6f^- fluorescence phenotype that was confirmed by their deficiency in cytochrome *f* and their inability to grow on minimum medium. The other two *TCA1* members of the tetrads showed a Cyt b_6f^+ phenotype, accumulated cytochrome *f*, and were phototrophic (Figure 3B). In five representative tetrads, none of the *tca1-1* progeny grew on antibiotic-supplemented medium (50 μg ml^{-1} spectinomycin plus 4 μg ml^{-1} streptomycin), in contrast to the *TCA1* progeny that were antibiotic resistant (Figure 3C). We took the loss of antibiotic resistance in the

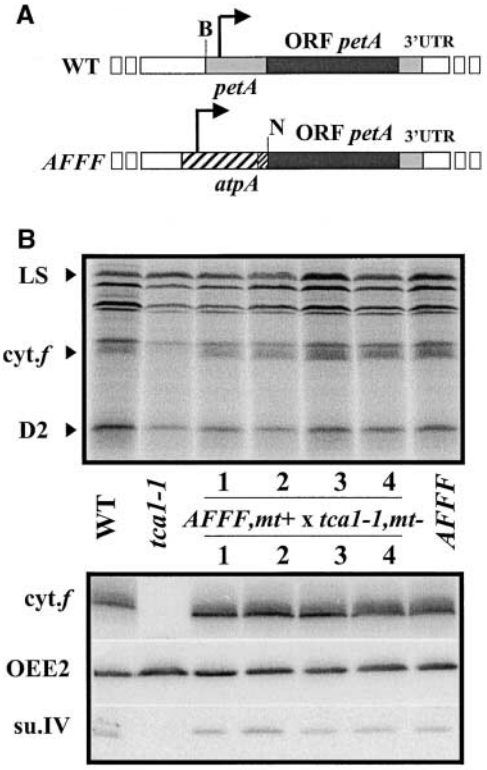

FIGURE 2.—Cytochrome *f* synthesized under the control of *atpA*-5′ UTR no longer requires the wild-type TCA1 factor. (A) Maps of the *petA* gene in wild-type and *AFFF* strains (CHOQUET *et al.* 1998). Relevant restriction sites (B, *Bgl*II; N, *Nco*I) are indicated. The heavily hatched box in the 5′ region of *atpA* denotes the sequence encoding the first 25 amino acids from the α-subunit of the ATP synthase complex. (B) Top, newly synthesized cytochrome *f* detected by pulse-labeling experiments in wild-type parental strains *AFFF* and *tca1-1* and in progeny of a representative tetrad from the cross *AFFF, mt+* × *tca1-1, mt−*. Bottom, accumulation of cytochrome *f*, subunit IV, and OEE2 as a loading control, in the same strains, detected with specific antibodies.

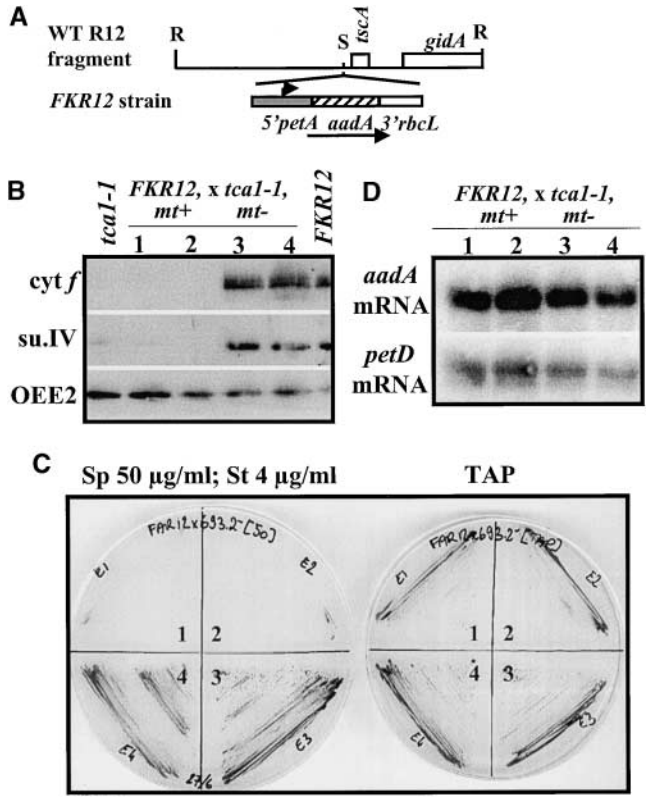

FIGURE 3.—Expression of the *FKR12* reporter gene driven by the *petA*-5′ UTR is dependent upon the wild-type TCA1 factor. (A) Maps of the R12 fragment in wild-type and *FKR12* strains (CHOQUET *et al.* 1998). Positions of known genes and relevant restriction sites are indicated (S, *Stu*I; R, *Eco*RI). (B) Identification of the *tca1* members lacking cytochrome *f* accumulation in the progeny of a representative tetrad from the cross *FKR12, mt+* × *tca1-1, mt−*. (C) Analysis of antibiotic resistance conducted on the same tetrad (Sp, spectinomycin; St, streptomycin). (D) Accumulation of the chimeric *FKR12* mRNA in the four progeny of the tetrad determined with an *aadA* probe.

tca1-1 context as indicative of a lack of translation of the *FKR12* chimeric mRNA. The 5′ UTR of the *petA* mRNA was thus sufficient to confer TCA1 sensitivity to a reporter gene. This observation demonstrates that the mRNA target for TCA1 is located within the *petA*-5′ UTR.

Reversion strategy of *tca1* mutants: The molecular identification of TCA1 partners in the control of *petA* translation should be tractable by generating extragenic suppressor mutations either in the chloroplast or in the nuclear genome of a primary *tca1* mutation. Therefore we undertook a search for chloroplast revertants, aimed at the characterization of *cis*-acting elements controlling translation within the *petA*-5′ UTR, and a search for nuclear revertants to identify other possible nuclear gene products interacting with the TCA1 factor.

All *tca1* mutants showed weak frequencies of spontaneous reversion, ranging from $<10^{-9}$ to 3.10^{-7}, as indicated in the diagonal of Table 1. A search for phototrophic revertants induced by mutagenesis was conducted most extensively with *tca1-1, mt+* and *tca1-2, mt+* strains. We used EMS and UV, which cause preferentially nuclear mutations, and FdUrd, which is known to reduce chloroplast polyploidy and induce chloroplast mutations preferentially (WURTZ *et al.* 1977, 1979). From 49 treatments, 21 independent revertants were isolated on the basis of their restored growth in phototrophic conditions under an 80 µE m^{-2} sec^{-1} illumination (Table 2). As illustrated in Figure 4, revertant strains recovered from 11 to 89% of the wild-type level of cytochrome *f* and accumulated subunit IV accordingly. True reversions could be excluded since none of the revertants recovered wild-type levels of cytochrome *f*. Consequently the suppressed phenotype could be most often distinguished from a wild-type phenotype by fluorescence tests in further crosses. We noted during our mutagenesis experiments that suppressor mutations from the UV-induced *tca1-1* mutant were most often found after UV treatments than after FdUrd treatments, while they were

TABLE 2

Isolation and analysis of revertants from *tca1* mutants

Original *tca1* strain	Mutagenic agent used for reversion	Frequency of treatments leading to reversion	Revertants strains	Reversion: nuclear (N) or chloroplastic (Cp)[d]	Frequency of Cyt b_6f^- progeny in WT cross[h]
tca1-1 mt+ (UV)[a]	None	2/7	*r1*	N	0/32 (<6.2 cM)
			r6	N	ND
	1 mM FdUrd	2/15	*r4*	N	0/6 (<33 cM)
			r5	N	0/22 (<9 cM)
	0.1 mM FdUrd + 2% EMS[b]	1/1	*r2*	N	0/33 (<6 cM)
	2% EMS (G)[c]	1/1	*r3*	N	0/65 (<3.1 cM)
	UV	5/7	*r7*	N	0/51 (<3.9 cM)
			r8	N	0/80 (<2.5 cM)
			r9	N	0/81 (<2.4 cM)
			r10	N	ND
			r11	[e]	
tca1-2 mt+ (FdUrd)[a]	None	0/6			
	1 mM FdUrd	7/14	*r1*	Cp	
			r4	N	0/116 (<1.7 cM)
			r5	N	0/60 (<3.3 cM)
			r6	[e]	
			r7	[f]	
			r8	N	0/80 (<2.5 cM)
			r9	N	0/64 (<3.1 cM)
	0.1 mM FdUrd + 2% EMS[b]	1/1	*r2*	N	0/49 (<4.1 cM)
	2% EMS (G)[c]	0/1			
	UV	1/7	*r3*	[g]	
tca1-3 mt+ (UV)[a]	1 mM FdUrd	1/1	*r1*	N	0/29 (<6.9 cM)
tca1-4 mt+ (UV)[a]	1 mM FdUrd	0/1			

[a] Mutagenic agent used to obtain the original *tca1* allele.

[b] Pretreatment with FdUrd followed by EMS treatment on vegetative cells.

[c] EMS treatment on gamete (G) cells.

[d] Genetic determinism of the suppression event determined from backcrosses of the revertant *mt+* strains with *mt−* strains bearing the original *tca1* mutation: nuclear determinism (N) for a mononuclear Mendelian segregation and chloroplast determinism (Cp) for a uniparental transmission by the *mt+* parental strain.

[e] The reversion phenotype was not transmitted to the progeny in the backcross with *tca1, mt−* strain (22 and 52 meiotic products tested for *tca1-1 r11* and *tca1-2 r6*, respectively).

[f] Poor spore viability (<10%).

[g] No preferential transmission of the revertant phenotype was observed excluding Cp inheritance. Three types of tetrads were observed (on 16 tested), indicating more than one nuclear suppression event.

[h] For revertants due to monogenic nuclear suppression, the number of meiotic products of Cyt b_6f^- phenotype are reported to the number of all meiotic products from tetrads obtained in crosses between revertant strains and the wild-type strain. Maximum genetic distances between forward and reverse mutations are indicated in parentheses. They are calculated as in Table 1, assuming that a recombinant progeny with the reverse mutation is phototrophic (if it had a Cyt b_6f^- phenotype, the values would be one-half of those indicated). ND, not determined.

most often obtained after FdUrd treatments than after UV treatments from the FdUrd-induced *tca1-2* mutant (Table 2). Thus, the molecular mechanisms at work on the nuclear genome in FdUrd mutagenesis are different from those of UV.

Genetic analysis of revertant strains: To determine the genetic origin of the suppression events, each *mt+* revertant strain was backcrossed with a *mt−* strain bearing the original *tca1* mutation. The suppressed and Cyt b_6f^- phenotypes should segregate 2:2 if the suppression event is due to a single nuclear mutation whereas the suppressed phenotype should be transmitted to the whole progeny if it is due to a chloroplast mutation (Table 2). For 16 revertants, a 2:2 segregation of the suppressed and Cyt b_6f^- phenotypes was observed either in tetrads or statistically on batches of meiotic products. This was indicative of a single nuclear mutational event at the origin of the reversion. However, for some of these

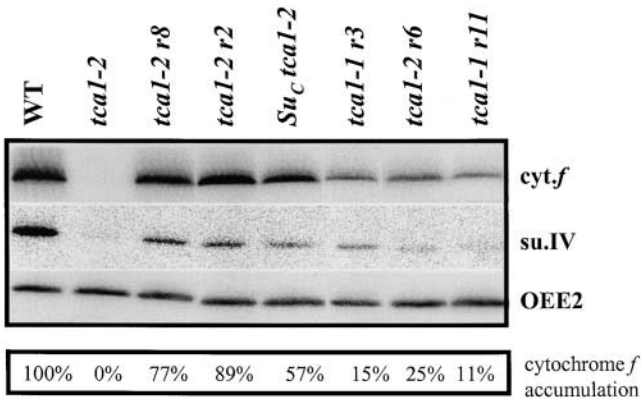

FIGURE 4.—Cytochrome *f* accumulation in revertant strains. Cytochrome *f* and subunit IV accumulation in the various revertant strains obtained either from *tca1-1* or *tca1-2* mutant strains was detected using specific antibodies. OEE2 accumulation is presented as a loading control. Estimated percentage of cytochrome *f* accumulation in each strain is presented at the bottom.

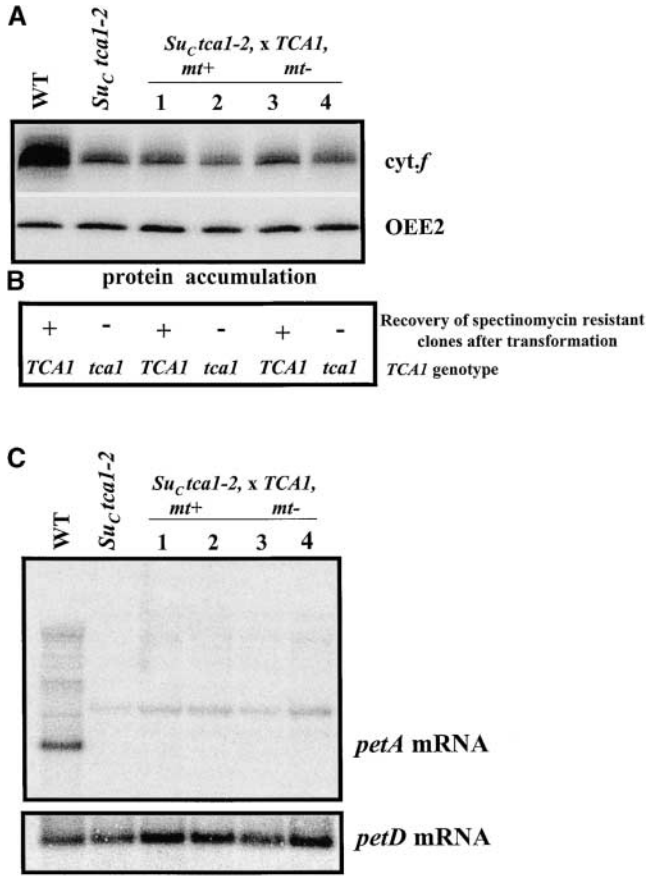

FIGURE 5.—The Su_C chloroplast suppressor allows a *TCA1*-independent translation of *petA*. (A) Cytochrome *f* accumulation in the progeny of one representative tetrad from the cross Su_C, *tca1-2, mt+* × *TCA1, mt−* and in the parental strains. OEE2 is presented as a loading control. (B) *TCA1* genotype was determined from the recovery or not of spectinomycin-resistant transformants (+ for *TCA1*, − for *tca1*) after biolistic transformation from each meiotic product by the *FKR12* chimeric gene. (C) *petA* mRNA accumulation in the same tetrad progeny and in the parental strains. *petD* mRNA accumulation is presented as a loading control.

revertant strains, we noted that the level of cytochrome *f* varied over time as well as among the progeny after a cross. For example, cytochrome *f* accumulated to ~50% of the wild-type level in the original *tca1-1 r3, mt+* strain but dropped down to ~15% in a *mt−* progeny from a *tca1-1 r3, mt+* × *tca1-1, mt−* cross. From a further backcross of this *tca1-1 r3, mt−* strain with the original *tca1-1, mt+* strain, six meiotic products with a suppressed phenotype showed variable cytochrome *f* accumulation ranging from 15 to 30% of the wild-type amount. These variations point to the possible instability of the TCA1 mutated factor whose steady-state concentration may then depend on the genetic background in each strain.

Fourteen of these 16 nuclear revertants due to monogenic nuclear suppression were crossed with the wild type to test the linkage of the suppressor and *tca1* mutations. No meiotic progeny yielded recombinant clones of Cyt b_6f^- phenotype, indicating low genetic distances between forward and reverse mutations (Table 2). Thus, all these 14 nuclear suppressor mutations were tightly linked to the original *tca1* mutations as one would expect for intragenic suppressors. We thus most likely obtained 14 new alleles of *TCA1* but got no genetic evidence for additional *TCA* nuclear genes.

Four suppressors were not analyzed further because they failed to show either predicted monogenic Mendelian inheritance or chloroplast uniparental inheritance or they showed poor spore viability after meiosis.

In a backcross of the FdUrd-induced *r1* revertant from *tca1-2, mt+* strain with a *tca1-2, mt−* strain, the whole progeny from 10 tetrads exhibited the suppressed phenotype. We then used a *mt−* clone from the progeny to perform a symmetric backcross with a *tca1-2, mt+* mutant strain. This cross yielded only clones of Cyt b_6f^- phenotype among the progeny from 6 tetrads. Thus, the suppressed phenotype was transmitted only by the *mt+* parental strain, indicative of a chloroplast suppressor mutation.

Characterization of the chloroplast suppressor event in the *r1* revertant: The *r1* revertant strain of *tca1-2* strain contained both the original *tca1-2* nuclear mutation and a chloroplast suppressor mutation that we called Su_C. It was crossed to the wild type to recover progeny carrying the suppressor mutation in a wild-type nuclear context. From two tetrads, the two *TCA1* progeny were distinguished from the *tca1-2* members by transformation with the *FKR12* chimeric gene (Figure 5B): the chimeric construct conferred spectinomycin resistance to the *TCA1* strains, while *tca1-2* mutation prevented its expression (see Figure 3). However, cytochrome *f* accumulated to about the same amount in the whole tetrads (Figure 5A) and in the parental *r1* revertant *mt+* strain. In this experiment cytochrome *f* accumulated to ~20% of the

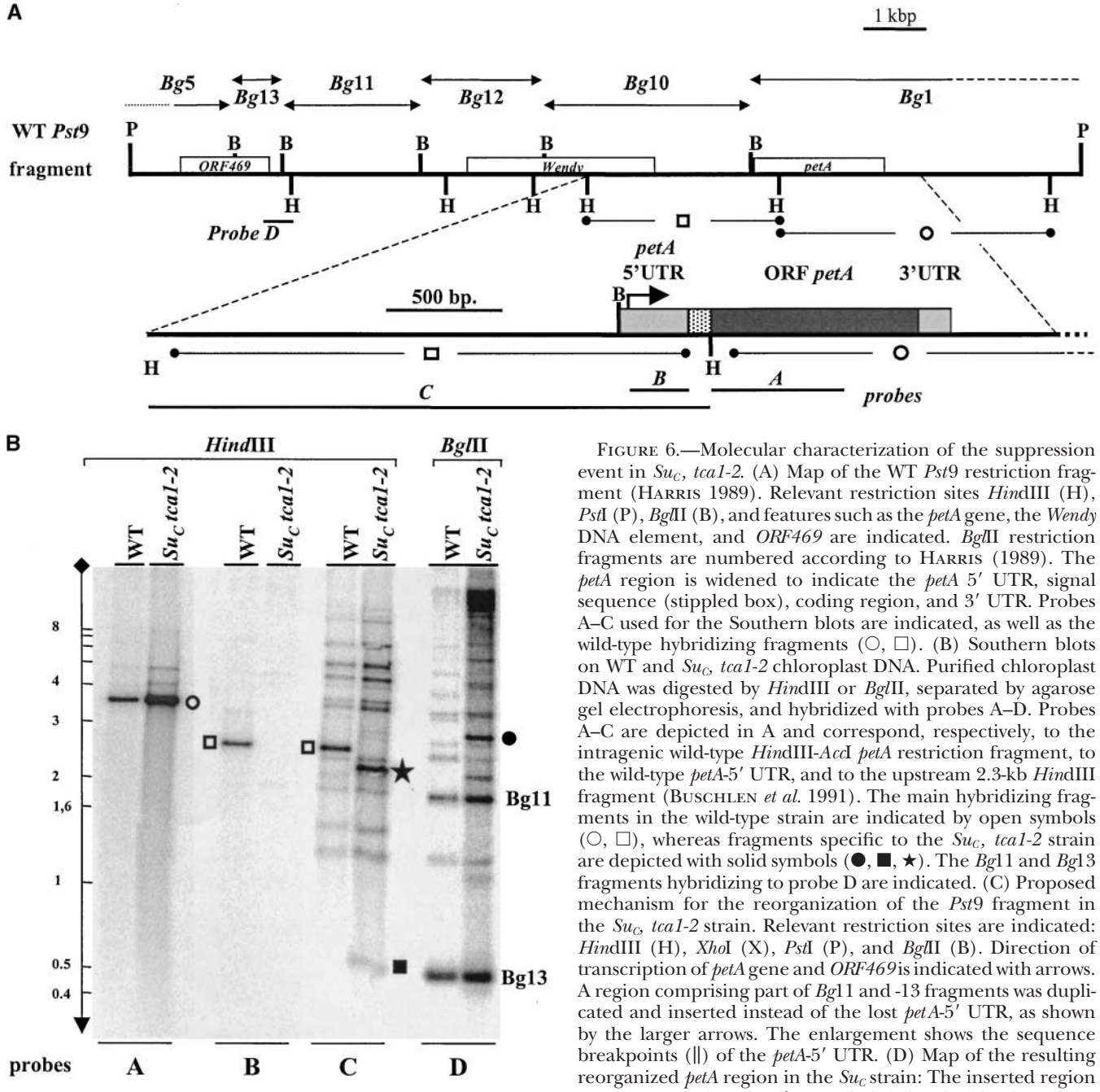

FIGURE 6.—Molecular characterization of the suppression event in Su_C, *tca1-2*. (A) Map of the WT *Pst*9 restriction fragment (HARRIS 1989). Relevant restriction sites *Hin*dIII (H), *Pst*I (P), *Bgl*II (B), and features such as the *petA* gene, the *Wendy* DNA element, and *ORF469* are indicated. *Bgl*II restriction fragments are numbered according to HARRIS (1989). The *petA* region is widened to indicate the *petA* 5′ UTR, signal sequence (stippled box), coding region, and 3′ UTR. Probes A–C used for the Southern blots are indicated, as well as the wild-type hybridizing fragments (○, □). (B) Southern blots on WT and Su_C, *tca1-2* chloroplast DNA. Purified chloroplast DNA was digested by *Hin*dIII or *Bgl*II, separated by agarose gel electrophoresis, and hybridized with probes A–D. Probes A–C are depicted in A and correspond, respectively, to the intragenic wild-type *Hin*dIII-*Acc*I *petA* restriction fragment, to the wild-type *petA*-5′ UTR, and to the upstream 2.3-kb *Hin*dIII fragment (BUSCHLEN *et al.* 1991). The main hybridizing fragments in the wild-type strain are indicated by open symbols (○, □), whereas fragments specific to the Su_C, *tca1-2* strain are depicted with solid symbols (●, ■, ★). The *Bg*l1 and *Bg*l3 fragments hybridizing to probe D are indicated. (C) Proposed mechanism for the reorganization of the *Pst*9 fragment in the Su_C, *tca1-2* strain. Relevant restriction sites are indicated: *Hin*dIII (H), *Xho*I (X), *Pst*I (P), and *Bgl*II (B). Direction of transcription of *petA* gene and *ORF469* is indicated with arrows. A region comprising part of *Bg*l1 and -l3 fragments was duplicated and inserted instead of the lost *petA*-5′ UTR, as shown by the larger arrows. The enlargement shows the sequence breakpoints (‖) of the *petA*-5′ UTR. (D) Map of the resulting reorganized *petA* region in the Su_C strain: The inserted region contains a $tRNA_{Val}$, the 5′ UTR (lightly hatched box), and the first 93 residues (darkly hatched box) of *ORF469* fused to the last 19 amino acids of the *petA* transit peptide with the downstream coding sequence. The amino acid sequence of the fusion region is shown at the bottom with amino acids from *ORF469* written in boldface type. The hybridizing fragments specific to the Su_C strain (●, ■, ★) and the *Hin*dIII-*Hin*fI fragment corresponding to probe D used in Southern blots are indicated.

wild-type level while it accumulated to ~50% in a first experiment (Figure 4; a similar situation was discussed above with *tca1-1 r3* mutant strains). Thus, the chloroplast mutation Su_C allowed only a limited accumulation of cytochrome *f*, even in a wild-type nuclear context. Interestingly, the *petA* mRNA accumulating in the *r1* revertant strain and in the four members of the tetrad was of larger size than the wild-type *petA* mRNA and its accumulation was reduced (Figure 5C).

To determine the specificity of the chloroplast suppressor Su_C, the Su_C, *tca1-2, mt+* revertant strain was crossed with *tca1-1, tca1-3*, and *mca1-792* mutant strains. The whole tetrad progeny from these crosses (10, 3, and 6 tetrads tested, respectively) displayed the suppressed phenotype in fluorescence tests (data not shown). Furthermore, cytochrome *f* accumulated to the same level as in the parental Su_C, *tca1-2* strain in two tetrads analyzed (data not shown). Thus, the chloroplast Su_C muta-

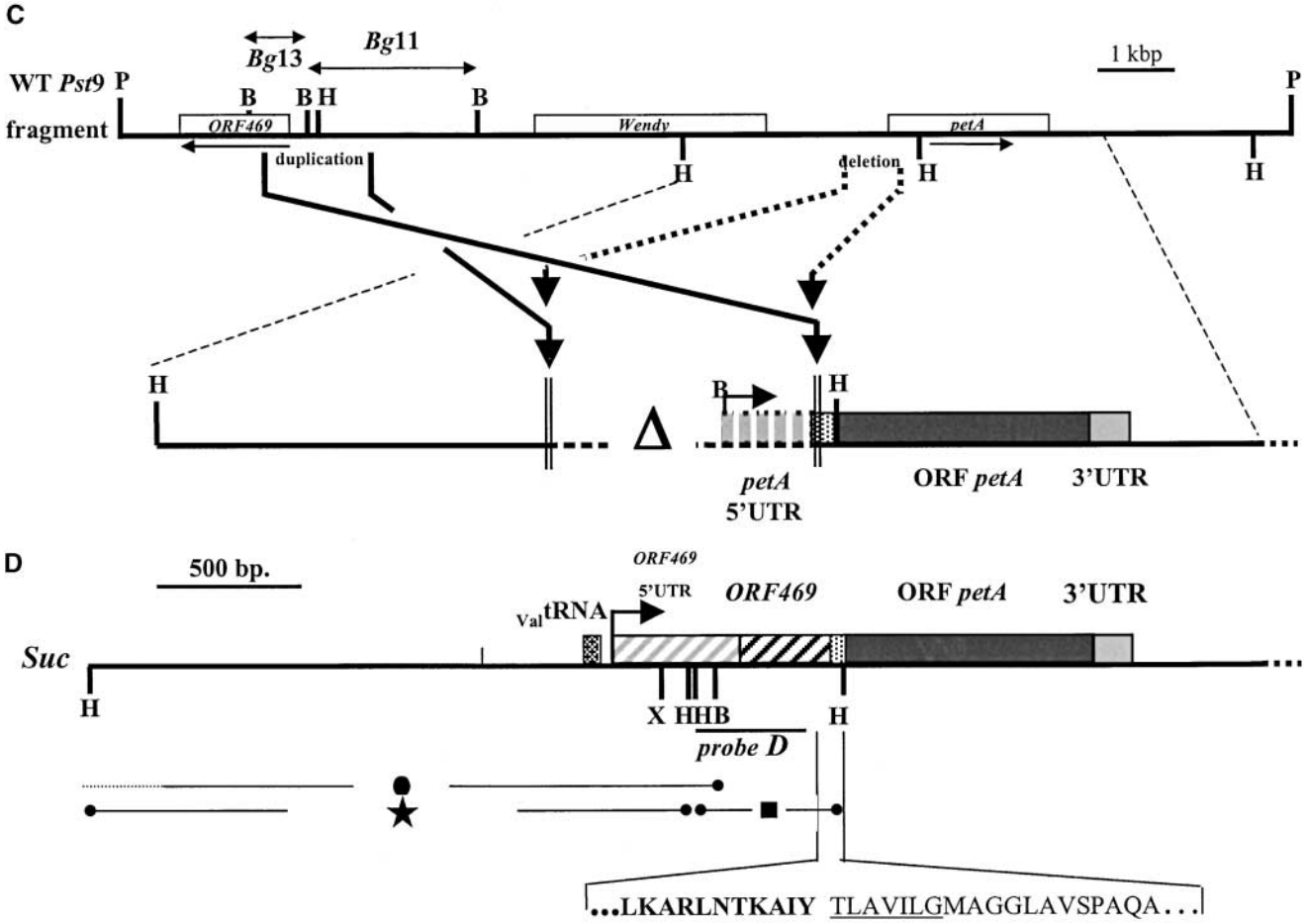

FIGURE 6.—*Continued.*

tion suppressed other *tca1* or *mca1* mutations. We then investigated whether the CES process mentioned in the Introduction still occurred in this chloroplast revertant strain. We introduced a deletion of the *petB* gene encoding cytochrome b_6 by biolistic transformation of the chloroplast genome from *r1* revertant strain with plasmid Δ*petB* (see MATERIALS AND METHODS). While cytochrome b_6 deletion mutants exhibit a 10-fold reduction of cytochrome *f* expression (KURAS and WOLLMAN 1994), the strain Su_C, Δ*petB, tca1-2* displayed no reduction of cytochrome *f* expression compared to the original Su_C, *tca1-2* strain (data not shown).

Thus, the chloroplast suppression turned out to confer a TCA1-, MCA1-, and CES-independent expression of the *petA* gene. Since the 5′ UTR of *petA* mRNA is the target of both factors and of the CES process, this suggested, together with the larger size of the *petA* mRNA accumulated in the Su_C strains, that the 5′ UTR region was deeply altered in these strains.

Molecular characterization of the chloroplast suppression event: To characterize the putative rearrangement in the *petA* region in Su_C, *tca1-2* strains, *Hin*dIII and *Bgl*II digests of purified chloroplast DNA isolated from the mutant and wild-type strains were compared by Southern blot analysis. The 3.5-kb *Hin*dIII fragment (○, Figure 6A), containing the coding sequence for mature cytochrome *f* and the downstream regions detected with probe A, remained unaffected, as expected from the accumulation of a functional cytochrome *f* (Figure 6B, lane A). Conversely, probe B, corresponding to the *petA*-5′ UTR, clearly showed the absence of any hybridizing fragment in the Su_C, *tca1-2* strain (Figure 6B, lane B). Thus, the *petA*-5′ UTR, the target of TCA1 and MCA1 factors and of the CES process, has been lost as a result of the mutation that leads to the suppressed phenotype. This was confirmed by hybridization with probe C, corresponding to the *Hin*dIII fragment upstream of the cytochrome *f* coding sequence (Figure 6A). This 2.3-kb fragment (□) was not detected any longer in the Su_C, *tca1-2* strain, which contained instead two new hybridizing fragments of 2.0 (★) and 0.5 kb (■; Figure 6B, lane C). The 2.0-kb *Hin*dIII fragment was subcloned into pUN121 and sequenced. The characterization of the rearrangement was completed by sequencing a PCR product amplified using oligonucleotides primers derived from the sequence of the cloned fragment and from the *petA* coding region (see MATERIALS AND METHODS). In the Su_C, *tca1-2* strain, the deleted

region of the *petA*-5′ UTR is replaced by 1.1 kb of unidentified sequences. To address the origin of this sequence, Southern blots of chloroplast *Bgl*II digestion products were probed with a *Hin*dIII-*Hin*fI fragment internal to the inserted region (probe D, see Figure 6D). As depicted in Figure 6B, lane D, this probe hybridized to the *Bgl*II fragments *Bg*13 and *Bg*11 in the wild-type and Su_C, *tca1-2* strains. This assignment was consistent with other features of the sequence of the insert such as the presence of a $tRNA_{val}$ and of *Xho*I and *Bgl*II restriction sites (Harris 1989). A final confirmation of the identity between the sequence of this fragment and that of the *Bg*13-*Bg*11 region came from the sequence data kindly communicated by D. Stern and J. Maul in the frame of the Chlamydomonas chloroplast sequencing project. In the Su_C, *tca1-2* strain, probe D strongly hybridized to a new band of 2.6 kb (●). The origin of this new band is shown diagrammatically in Figure 6C. Two other faint hybridizing fragments of 2- and 1-kb size are of unknown origin. As confirmed by Southern blots using other restriction enzymes (data not shown), the *Bg*11 and *Bg*13 fragments remained unaffected in the Su_C, *tca1-2* strain compared to the wild-type strain. Thus, the rearrangement results from a duplication of this region.

This rearrangement leads to a chimeric gene encoding a new protein made of the cytochrome *f* sequence, including 19 of the 31 residues from its transit peptide fused in frame with 93 amino acids corresponding to the N-terminal part of a 469-amino-acid open reading frame (*ORF469*) present in the *Bg*13-*Bg*11 region (Figure 6C). The fusion preserves the 7-amino-acid hydrophobic core of the cytochrome *f* transit peptide described by Smith and Kohorn (1994) and the AQA target sequence for the lumenal peptidase (Buschlen *et al.* 1991; Kuras *et al.* 1995a; Baymann *et al.* 1999; Figure 6D). Thus, the mature product from the chimeric gene is identical to wild-type cytochrome *f* (Figure 5A). This chimeric *petA* gene is now under the control of *cis*-acting sequences normally required for the expression of *ORF469*, suggesting that this putative ORF is expressed. The modification of the 5′ UTR of the *petA* gene explains the accumulation to ~5% of the wild-type level of a transcript of higher molecular weight as depicted in Figure 5C.

Study of the CES process in strains with partially restored TCA1 activity: As mentioned in the Introduction, the CES process for cytochrome *f* is likely to operate through a ternary effector that would interact with the *petA*-5′ UTR. This effector should modulate the translational efficiency of *petA* mRNA, depending on its binding to unassembled cytochrome *f*. The above experiments indicated that the TCA1 factor and the CES process both involved as a target the *petA*-5′ UTR. We wondered whether the partially restored translation of cytochrome *f* in a nuclear *tca1*-suppressed strain, which should express a mutated version of the TCA1 factor, still showed sensitivity to the CES process. To this end

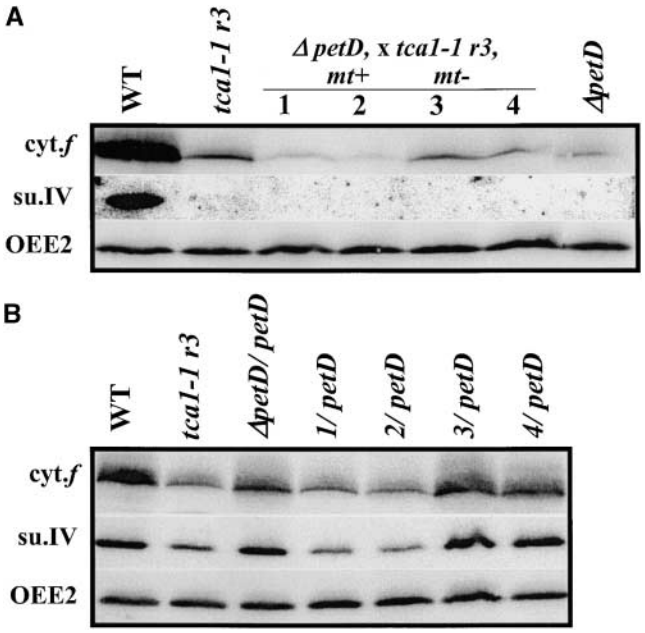

Figure 7.—Cytochrome *f* accumulation in the progeny of a representative tetrad from the cross Δ*petD, mt*+ × *tca1-1 r3, mt*−. (A) Cytochrome *f* and subunit IV accumulations, detected using specific antibodies, are compared in the wild-type and parental strains and in a representative tetrad progeny from the cross Δ*petD, mt*+ × *tca1-1 r3, mt*−. OEE2 accumulation is presented as a loading control. (B) Accumulation of cytochrome *f* and subunit IV was similarly monitored in strains obtained by biolistic transformation of the strains depicted in A with the wild-type *petD* gene. OEE2 accumulation is presented as a loading control.

we studied the CES process in a revertant strain carrying a leaky *TCA1* allele, *tca1-1 r3, mt*−, which accumulated ~15% of wild-type cytochrome *f* (Figures 7A and 8A). We investigated the expression of the *petA* gene in tetrads obtained from crosses between *tca1-1 r3, mt*− and two types of chloroplast transformants in which cytochrome *f* translation was either repressed or enhanced.

The first type of cross involved the Δ*petD, mt*+ strain and consequently displays a cytochrome *f* synthesis reduced by ~90% as illustrated in Figure 7A. We analyzed four tetrads from a Δ*petD, mt*+ × *tca1-1 r3, mt*− cross. The whole progeny inherited the Δ*petD* deletion and lacked subunit IV, as illustrated in Figure 7A. We observed a Mendelian segregation of cytochrome *f* accumulation: two members of the tetrads accumulated ~15% of wild-type cytochrome *f*, while this accumulation was reduced to ~3% in the other two members of the tetrad (see Figure 7A). We observed changes in the *petA* mRNA levels among these various strains (data not shown). However, as previously reported (Sakamoto *et al.* 1994; Choquet *et al.* 1998) we found no direct quantitative relation between *petA* mRNA levels and the rates of cytochrome *f* synthesis. To determine the genotype of that tetrad progeny, the wild-type *petD* gene was reintroduced by biolistic transformation using plasmid

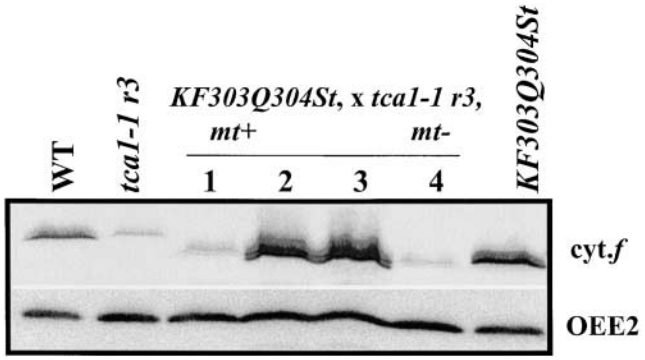

FIGURE 8.—Cytochrome *f* accumulation in the progeny of a representative tetrad from the cross *KF303Q304St, mt+* × *tca1-1 r3, mt−*. Accumulation of cytochrome *f* and OEE2 (as a loading control) is detected by specific antibodies in the wild type, in the parental strains *KF303Q304St* and *tca1-1 r3*, and in the progeny of the cross *KF303Q304St, mt+* × *tca1-1 r3, mt−*.

pWQ in each member of the representative tetrad presented in Figure 7A. Accumulation of cytochrome *f* in the resulting transformant strains is shown in Figure 7B. The third and fourth members of the tetrad had the wild-type *TCA1* allele since they exhibited wild-type levels of cytochrome *f* after transformation, while the first and second members had inherited the partially active *tca1-1 r3* allele since their transformants accumulated cytochrome *f* to the same level as the parental strain *tca1-1 r3, mt−*. Comparison of the state of cytochrome *f* accumulation in the first and second members of the tetrad before and after reintroduction of the *petD* gene expressing subunit IV shows that the CES process still occurred in cells bearing the leaky *tca1-1 r3* allele, with a repressed expression of cytochrome *f* in the absence of subunit IV.

For the second type of cross, we used the *KF303-Q304St, mt+* strain that expresses a mutated version of cytochrome *f* deleted for the last 14 residues of the C-terminal domain. As shown in Figure 8, this strain behaves similarly to the $FK_{283}St$ strain lacking the whole C-terminal domain (KURAS *et al.* 1995b): the mutated cytochrome *f* is overexpressed threefold, irrespective of the presence or absence of its assembly partners. Two tetrads were analyzed from the cross *KF303Q304St, mt+* × *tca1-1 r3, mt−*. All progeny displayed the mutated version of cytochrome *f* as evidenced by its faster electrophoretic migration pattern upon SDS-PAGE. Two members of the tetrads overexpressed cytochrome *f* to the same extent as the parental *KF303Q304St, mt+* strain (Figure 8, members 2 and 3 of a representative tetrad). The other two members of the progeny accumulated the mutated version of cytochrome *f* to the same level as did wild-type cytochrome *f* in the parental *tca1-1 r3, mt−* strain (Figure 8, members 1 and 4). Thus, clones 2 and 3 had a wild-type version of TCA1 while clones 1 and 4 bore the mutated TCA1 factor. According to the molecular model we have proposed for the CES process (CHOQUET *et al.* 1998), TCA1 (either in its wild-type or mutated form) is no longer trapped by unassembled cytochrome *f* since the regulatory motif carried by the C-terminal domain involved in this mechanism is deleted in the *KF303Q304St, mt+* strain. The wild-type or mutated versions of TCA1 are therefore entirely available to promote *petA* mRNA translation, but the reduced activity of the mutated TCA1 factor turned out to be limiting in the expression of cytochrome *f*.

DISCUSSION

TCA1 is a nuclear-encoded activator required for the initiation of translation of *petA* mRNA: We performed a genetic identification of a nuclear factor specifically required for the expression of the chloroplast *petA* gene encoding cytochrome *f*, a major subunit of cytochrome b_6f complexes from the thylakoid membranes. Mutations in the *TCA1* gene still allow a reduced but significant accumulation of the *petA* mRNA but impair the synthesis of cytochrome *f*. The lower stability of the untranslated *petA* mRNA in a *tca1* context correlates with a block at the stage of translation initiation since we showed, using chimeric genes, that the target for TCA1 is located entirely in the 5′ UTR region of the *petA* transcript. As the seven *TCA1* mutations presented in this study were recessive, we conclude that the TCA1 product acts as a specific translational activator and not as a translational repressor. The reason for the destabilization of *petA* mRNA in *tca1* mutants is unclear. In a *tca1* mutant nuclear context, the accumulation of the *petA* messenger is reduced (Figure 1B) but not that of the chimeric *FKR* transcript (Figure 3D). A comparative analysis of several other studies shows that there is no simple relationship between translation and stability of chloroplast mRNAs in Chlamydomonas. The *tda1-F54* nuclear mutant strain, impaired in the translation of the α-subunit of the ATP synthase complex, showed a threefold increase of the level of *atpA* mRNA (DRAPIER *et al.* 1992), while the *tbc1-F34* and *tbc2-F64* mutants, lacking translation initiation of *psbC* mRNA, showed no changes at the transcript level (ROCHAIX *et al.* 1989). From the analysis of translational defects for the *psaB* chloroplast mRNA it is tempting to suggest that a translational block after initiation may protect mRNAs from degradation whereas impaired initiation would compromise mRNA steady-state accumulation: indeed, there was a fivefold reduction in the *tab1-F15* mutant specifically defective in translation initiation (STAMPACCHIA *et al.* 1997). In contrast premature arrest of translation of the same *psaB* mRNA, because of a frameshift or because of addition of lincomycin, caused an increase in the transcript level (XU *et al.* 1993). However, in the absence of initiation of translation, the lifetime of a chloroplast mRNA certainly depends on determinants downstream of the 5′ UTR since, in contrast to the resident *psaB* mRNA, a chimeric

5′*psaB-aadA*-3′*rbcL* mRNA that contains the target sequence of the TAB1 factor accumulated to similar levels in *tab1* and *TAB1* genetic nuclear contexts (Stampacchia *et al.* 1997).

Are there other TCA factors? The number of nuclear genes required for the achievement of a given step in the expression of one chloroplast gene in *C. reinhardtii* is probably higher than estimated from our current knowledge. In most cases, these nuclear genes were identified by a single mutant allele, indicating that we are far from genetic saturation and that other genes remain to be discovered. The situation is widely different for cytochrome *f*. Although we used several mutagenic agents to carry out a large-scale screening of cytochrome b_6f-deficient mutants, we identified only a single nuclear gene, *TCA1*, controlling cytochrome *f* translation, out of seven independent mutants deficient in cytochrome *f* translation. The five other nuclear mutations responsible for a deficiency of cytochrome *f* synthesis lacked mature *petA* mRNA and were all located in another unlinked gene called *MCA1*, identified for the first time by the *MΦ11* mutation (Gumpel *et al.* 1995; J. Girard-Bascou and Y. Choquet, unpublished results). Thus, the 12 selected nuclear mutants deficient in cytochrome *f* synthesis presented in this study were mutated either in *TCA1* or *MCA1* genes. Moreover, the 14 nuclear *tca1* suppressors that we could further analyze in crosses were most likely intragenic suppressors exhibiting a broad range of cytochrome *f* accumulation. This extensive search for genes involved in cytochrome *f* synthesis suggests that there are indeed only two genes, one controlling specifically the stability of *petA* mRNA (*MCA1* gene) and the other its translation (*TCA1* gene).

In yeast mitochondria, where extensive screens have been used, a limited number of specific translational activators have been characterized for each mitochondrial gene (for a review, see Fox 1996). For instance, translation of *COX3* mRNA requires three nuclear genes (*PET54, PET122,* and *PET494*) whose products form a complex. Only a single nuclear gene (*PET111*) was found to be required for *COX2* mRNA translation, the products of two nuclear genes (*CBS1* and *CBS2*) specifically activate translation of the *COB* mRNA, and one gene (*PET309*) is involved in *COX1* mRNA translation.

In maize, a nuclear gene, *Crp1*, has also been shown to affect cytochrome *f* translation (Barkan *et al.* 1994; Fisk *et al.* 1999). However, the phenotypes of the *crp1* mutant and the *tca1* mutant differ in their specificity. Besides its role in cytochrome *f* translation, *Crp1* is also involved in the processing of the dicistronic *petB-petD* transcript and translation of *petD* mRNA. The *Crp1* gene, cloned by transposon tagging, encodes a large soluble protein not associated with ribosomes, which is a component of a multisubunit complex in the chloroplast stroma (Fisk *et al.* 1999). *Crp1* homologs have been found in *Neurospora crassa* (*cya5*), *Saccharomyces cerevisiae* (*PET309*; Fisk *et al.* 1999), and recently in *C. reinhardtii* (*Tbc2*; for a review, see Zerges 2000). *Tbc2* is specifically required for translation of the chloroplast *psbC* mRNA, which encodes a subunit of PSII complex. Thus *Crp1* and *TCA1* genes are probably not related to one another.

Chloroplast suppression: Only one chloroplast suppression event was obtained after several FdUrd treatments of *tca1* mutant strains (Table 2). Furthermore, while chloroplast point mutations induced with FdUdr have been found on the 5′ UTR of *psbC* or *psaB* (Stampacchia *et al.* 1997; Zerges *et al.* 1997), the suppression event obtained in our study corresponds to an extensive chloroplast DNA rearrangement that led to the replacement of the whole 5′ UTR of *petA* by that of another gene, so that the *petA* gene expression in this strain is now independent of TCA1 and MCA1 and of the CES process. The rearrangement does not result from a reciprocal event as in a chloroplast revertant of a *C. reinhardtii* strain carrying a deletion in the 5′ UTR of the *petD* gene (Sturm *et al.* 1994; Higgs *et al.* 1998). Here, the *petA*-5′ UTR has been deleted from the chloroplast genome of the revertant strain, and a duplication of a 1.1-kb sequence located on the other side of the *wendy* transposon has been inserted immediately upstream of the *petA* coding region. The *Wendy* DNA element (see Figure 6A) is bordered by inverted repeats and several additional degenerate copies of repeated sequences in direct or inverted orientation (Fan *et al.* 1995), which may have played a role in the mutation event. However, no sequence homology has been detected at the breakpoints of the rearrangement, but illegitimate transpositional recombination without duplication of the element has been previously reported (Fan *et al.* 1995).

The rearrangement led to the disruption of the 31-amino-acid transit peptide of the apocytochrome *f* by deleting the first 12 amino acids but preserved the hydrophobic core required for the translocation of the protein (Smith and Kohorn 1994). Even though the molecular characterization of the suppression event gave no further insights about the target of TCA1 within the *petA* 5′ UTR, it led to the identification of a novel ORF of 469 amino acids, which is likely expressed since the chimeric gene resulting from the fusion between this ORF and the 5′-truncated *petA* gene is translated. This ORF being unaltered in the Su_C strain, we obtained no indication about its possible function. However, the C-terminal half of this ORF shares homologies (35–45% identity, 40–60% similarity) with the first 200 amino acids of chloroplast *rpoC1* or bacterial *rpoC* gene products. This could suggest that this ORF is essential for cell viability and explain why the suppressing event involved a duplication rather than a reciprocal event. Further characterization of this gene product is now in progress.

TCA1, a candidate to act as the ternary effector involved in the CES process that controls cytochrome *f* translation: The CES process that controls cytochrome *f* synthesis is an assembly-dependent autoregulation of translation that involves a regulatory motif carried by the C-terminal domain of the unassembled protein and

the 5′ UTR of the *petA* mRNA (Choquet *et al.* 1998). The CES process appears as a general regulation mechanism in the biogenesis of photosynthetic proteins in *C. reinhardtii* chloroplast, with at least one CES subunit present in each oligomeric protein of the thylakoid membrane: the α-subunit of the ATP synthase complex (Drapier *et al.* 1992), cytochrome *f* of the cytochrome b_6f complex (Kuras and Wollman 1994; Choquet *et al.* 1998), the PsaA reaction center subunit of PSI (Girard-Bascou *et al.* 1987; Stampacchia *et al.* 1997), the D1 and CP47 subunits of PSII (Bennoun *et al.* 1986; Erickson *et al.* 1986; Jensen *et al.* 1986; de Vitry *et al.* 1989), the large subunit of Rubisco (Khrebtukova and Spreitzer 1996; reviewed in Choquet *et al.* 1998; Wollman *et al.* 1999; Choquet and Vallon 2000). It is highly unlikely that all these CES subunits would have evolved specific RNA-binding domains, able to interact specifically with their own encoding mRNAs. Thus, a competitive binding of a ternary effector to the unassembled CES subunit and its mRNA is the most likely mechanism underlying the CES process. As discussed above, there is overwhelming evidence for the presence of chloroplast gene-specific translational activators of nuclear origin in *C. reinhardtii* (see Barkan and Goldschmidt-Clermont 2000; Zerges 2000 for reviews). Some of these nuclear factors could have been recruited for the CES process during evolution.

The present analysis of cytochrome *f* expression in a strain carrying a leaky allele of *TCA1* showed that the mutated TCA1 factor became limiting in the CES process, as expected from a ternary CES effector. First, cytochrome *f* expression could no longer be stimulated in that leaky *tca1* strain, even in the absence of the C-terminal regulatory motif of cytochrome *f*: The rate of synthesis of C-ter truncated mutated cytochrome *f* and wild-type cytochrome *f* remained similar in the *tca1* leaky strain, although these two protein versions showed a threefold difference in their expression levels in a wild-type nuclear context. Second, due to the absence of subunit IV, cytochrome *f* accumulation in the *tca1* leaky strain dropped from 15 to 3% of the wild-type level. The 5-fold repression observed in the presence of this *tca1* leaky allele compared to the 10-fold repression observed in a wild-type *TCA1* context can be attributed to the *tca1* leaky allele. This *tca1* leaky allele could correspond either to a drop in the concentration of a fully functional TCA1 factor or to a mutated TCA1 factor with altered function. Thus, the characteristics of the CES-mediated up- and downregulation of cytochrome *f* synthesis in the presence or absence of the *tca1* leaky allele are consistent with TCA1 being the CES ternary effector. The molecular identification of TCA1 should open the way to a refined characterization of the mechanism underlying the regulation of cytochrome *f* synthesis by the CES process.

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