

# Characterization of Tbc2, a nucleus-encoded factor specifically required for translation of the chloroplast *psbC* mRNA in *Chlamydomonas reinhardtii*

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Genetic analysis has revealed that the three nucleus-encoded factors Tbc1, Tbc2, and Tbc3 are involved in the translation of the chloroplast *psbC* mRNA of the eukaryotic green alga *Chlamydomonas reinhardtii*. In this study we report the isolation and phenotypic characterization of two new *tbc2* mutant alleles and their use for cloning and characterizing the *Tbc2* gene by genomic complementation. *TBC2* encodes a protein of 1,115 residues containing nine copies of a novel degenerate 38–40 amino acid repeat with

a quasiconserved PPPEW motif near its COOH-terminal end. The middle part of the Tbc2 protein displays partial amino acid sequence identity with Crp1, a protein from *Zea mays* that is implicated in the processing and translation of the chloroplast *petA* and *petD* RNAs. The Tbc2 protein is enriched in chloroplast stromal subfractions and is associated with a 400-kD protein complex that appears to play a role in the translation of specifically the *psbC* mRNA.

## Introduction

It is well established that the photosynthetic complexes of the thylakoid membrane have a dual genetic origin. Some subunits are encoded by chloroplast genes and translated on chloroplast ribosomes, whereas others are encoded by nuclear genes, translated as precursor proteins on cytosolic ribosomes, and imported into the chloroplast where they associate with their chloroplast-encoded partners, pigments, and cofactors to form functional complexes (Wollman et al., 1999). Thus, a coordinated expression of the nuclear and chloroplast genomes is essential for the proper biogenesis of the photosynthetic apparatus. Analysis of mutants of *Chlamydomonas reinhardtii* and higher plants deficient in photosynthetic activity has revealed that this coordination is complex and involves the functions encoded by a large number of nuclear loci (Goldschmidt-Clermont, 1998; Rochaix, 2001). Many of these are required for specific posttranscriptional steps of

chloroplast gene expression such as RNA stability, RNA processing, splicing, and translation. Recently, several of these factors have been characterized at the molecular level (Barkan and Goldschmidt-Clermont, 2000). A common feature of some of these proteins is the presence of tandem arrays of degenerate 34 (TPR) or 35 (PPR) amino acid repeats. One class, the TPR repeats, is present in Nac2 and Mbb1, two nucleus-encoded proteins of *C. reinhardtii* that are specifically required for the stable accumulation of the *psbD* and *psbB* mRNAs, respectively (Boudreau et al., 2000; Vaistij et al., 2000). Both of these proteins are associated with high molecular weight RNA–protein complexes. The other class, PPR repeats, has been identified in Crp1, a protein of maize that is required for the processing and translation of the *petA* and *petD* mRNAs. PPR repeats appear to have a structure similar to the TPR repeats, consisting of two short  $\alpha$ -helical regions (Fisk et al., 1999; Small and Peeters, 2000). Genes encoding proteins with the PPR motif are part of a large family in *Arabidopsis thaliana* (Small and Peeters, 2000).

Clues to the biochemical functions of some nucleus-encoded factors have come from identification of motifs or regions of homology these proteins share with enzymes known to be involved in RNA metabolism and other processes. For example, the splicing factor Raa2 (formerly called Maa2) resembles pseudouridine synthase and is required for the transsplicing of the second *psaA* intron in the chloroplast of *C. reinhardtii* (Perron et al., 1999). The splicing factor Crs2 of maize, re-

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Key words: chloroplast; translation; *Chlamydomonas*; photosynthetic mutant; protein complex

quired for the splicing of several plastid group II introns, is related to peptidyl-tRNA hydrolase enzymes (Jenkins and Barkan, 2001). Another splicing factor Raa3, which is involved in the splicing of the first *psaA* intron of *C. reinhardtii*, contains a short region of homology with pyridoxamine 5' phosphate oxidase (Rivier et al., 2001). In contrast, other factors do not resemble any known protein in the database. These include the *Chlamydomonas* Ac115 protein which is implicated in the translation elongation of the *psbD* mRNA (Rattanachaikunsopon et al., 1999).

The control of expression of the chloroplast genes encoding the major core photosystem (PS)II subunits D1, D2, P5, and P6 of *C. reinhardtii* has been studied intensively in recent years. Biochemical approaches have identified two nucleus-encoded proteins that are involved in the light activation of the translation of the *psbA* mRNA of *C. reinhardtii* (Danon and Mayfield, 1991): one is a 47-kD protein resembling polyA-binding proteins (Yohn et al., 1998), and the other is a protein disulfide isomerase that both appear to be under redox control (Kim and Mayfield, 1997; Fong et al., 2000; Trebitsh et al., 2000, 2001). Analysis of several nuclear mutants of *C. reinhardtii* deficient in PSII activity has revealed that each of these mutations identifies a nuclear locus that is required for the translation of the mRNA of one specific PSII subunit (Goldschmidt-Clermont, 1998). For example, translation of the *psbC* mRNA requires functions encoded by two nuclear loci, *TBC1* and *TBC2*. The *psbC* mRNA has the largest 5' untranslated region (UTR) of known chloroplast mRNAs in *C. reinhardtii*. It consists of 550 nucleotides, and acts as a target site for Tbc1 and Tbc2, strongly suggesting that these factors play a role in the initiation of translation (Zerges and Rochaix, 1994). A striking feature of the *psbC* 5'UTR is a large inverted repeat structure in its middle that is required for translation of the *psbC* mRNA. Mutations within this inverted repeat or deletion of the entire structure completely abrogate translation (Rochaix et al., 1989; Zerges and Rochaix, 1994; Zerges et al., 1997). A nuclear suppressor of these mutations has identified a third locus, *TBC3*, involved in the initiation of translation of *psbC* mRNA. This suppressor also reverses the translational defect caused by the *tbc1*, but not by the *tbc2*, mutation (Zerges et al., 1997). The two factors defined by *TBC1* and *TBC3* and the middle part of the *psbC* 5'UTR appear to interact functionally (Zerges et al., 1997). UV crosslinking studies with chloroplast extracts and the *psbC* 5'UTR have revealed an RNA binding activity in S100 fractions of a mutant strain carrying the *tbc2-F64* mutation, but not in similar fractions prepared from wild-type strains (Zerges and Rochaix, 1994). To understand the exact role of these factors, and in particular their interactions with the *psbC* mRNA, it is important to examine their genes and the products that these genes encode. As a first step toward this goal, we have cloned the *Tbc2* gene and characterized its product.

## Results

### Isolation and characterization of two new *TBC2* alleles

The *TBC1* and *TBC2* loci encode factors that are specifically required for the translation of the *psbC* mRNA. As only single mutant alleles of *TBC1* and *TBC2* have been reported, a

\*Abbreviations used in this paper: HA, hemagglutinin; PS, photosystem; UTR, untranslated region.

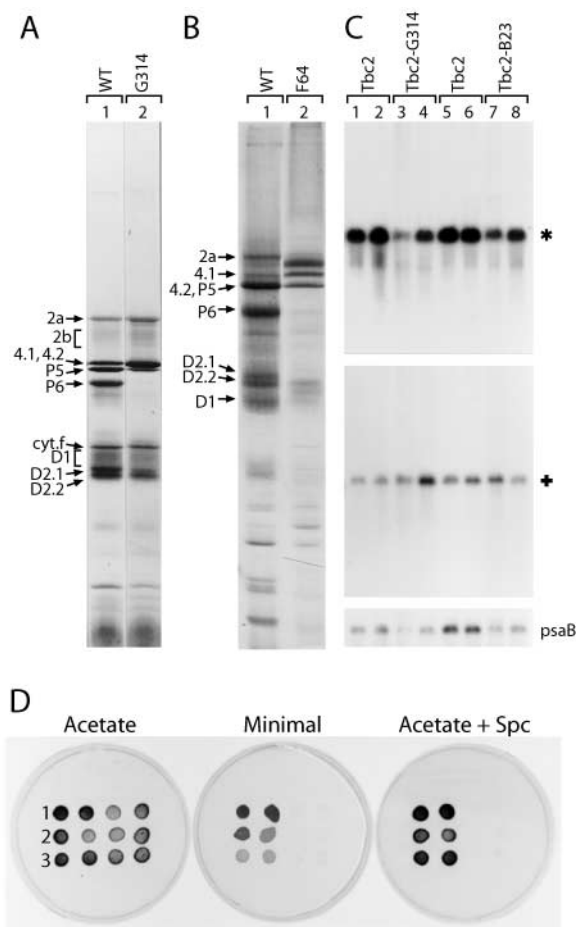


Figure 1. Characterization of two new alleles of the *TBC2* locus.

Pulse labeling of chloroplast-encoded polypeptides with [ $^{14}$ C]acetate for 45 min. (A) in wild-type (lane 1) and *G314* (lane 2), and for 5 min (B) in wild-type (lane 1) and *F64* (lane 2) in the presence of cycloheximide to inhibit protein synthesis in the cytosol. After pulse labeling, thylakoid membranes were isolated and the proteins fractionated on a 7.5–15% SDS-polyacrylamide gel (A) or a 12–18% SDS polyacrylamide gel with 8 M urea (B) (Chua and Bennoun, 1975). The pattern of protein synthesis in *B23* was similar to that of *G314* (unpublished data). The genes encoding the indicated polypeptides are: 2a, *psaB*; 2b, *psaA*; 4.1, *atpB*; 4.2, *atpA*; P5, *psbB*; P6, *psbC*; *cyt.f*, *petA*; D1, *psbA*; and D2, *psbD*. (C) RNA blot analysis of the *psbC* mRNA (\*) and the chimeric mRNA *psbC(WT)-aadA* (+) in RNA preparations from tetrads from cross 1 (*FuD50:psbC-aadA*, *mt*<sup>+</sup> × *G314*, *mt*<sup>-</sup>; lanes 1–4) and from cross 2 (*FuD50:psbC-aadA*, *mt*<sup>+</sup> × *B23*, *mt*<sup>-</sup>; lanes 5–8; Table I). RNA from the *TBC2*-bearing progeny were loaded in lanes 1, 2, 5, and 6, from the *tbc2-G314*-bearing progeny in lanes 3 and 4, and from the *tbc-B23*-bearing progeny in lanes 7 and 8. The level of *psaB* mRNA was used to standardize for the amount of RNA in each lane. (D) Growth of members of tetrads from a cross of *FuD50:psbC(WT)-aadA* (*mt*<sup>+</sup>) with *F64* (*mt*<sup>-</sup>), “1”; or *G314* (*mt*<sup>-</sup>), “2”; or *B23* (*mt*<sup>-</sup>), “3” on nonselective acetate containing medium (Acetate), on medium that selects for photosynthetic growth (Minimal), and on medium containing acetate and spectinomycin at 750  $\mu$ g ml<sup>-1</sup>.

genetic screen for new mutant alleles of these loci was performed. Among a number of mutants affected in a variety of processes, two mutants affected specifically in *psbC* mRNA translation were obtained: *G314* and *B23*. In vivo pulse labeling of thylakoid membrane proteins with [ $^{14}$ C]acetate for 45 min revealed that both mutants fail to synthesize the P6

polypeptide, which is encoded by *psbC* and is the homologue of CP43 in vascular plants (*G314*, Fig. 1 A; *B23*, unpublished data). These results were confirmed with a pulse-labeling time of 5 min using total cell proteins from *F64* (Fig. 1 B) containing a mutation that is allelic to those of *G314* and *B23* (see below). Similar to the *F34* and *F64* mutants previously described (Rochaix et al., 1989), which carry the *tbc1* and *tbc2* mutations, respectively, *G314* and *B23* accumulate wild-type levels of the *psbC* mRNA, as determined by RNA blot analysis (unpublished data; Fig. 1 C). When crossed to a strain containing the wild-type *TBC2* locus, *G314* and *B23* produced tetrads exhibiting a 2:2 segregation of PSII deficiency and wild-type photosynthesis, as determined by the analysis of fluorescence transients and tests for growth on minimal medium. More than 15 tetrads were examined for each cross, indicating that a single nuclear mutation in each strain is responsible for its PSII deficiency. However, we cannot exclude the occurrence of two tightly linked mutations (less than three map units apart). To determine whether the new mutations abolish translation of the *psbC* mRNA or cause a rapid degradation of the P6 polypeptide, we asked whether either mutation affects the expression of the *aadA* reporter gene driven from the *psbC* 5'UTR in the chimeric gene *psbC*(WT)-*aadA* (Zerges and Rochaix, 1994; Zerges et al., 1997). This chimeric gene was integrated into the chloroplast genome of the *Fud50(mt<sup>+</sup>)* strain, giving rise to a photosynthetic strain that was crossed to *G314* and *B23*. In each cross, six tetrads were examined. The chimeric gene was transmitted to all the progeny because the chloroplast genome is inherited uniparentally. Neither mutation affects the accumulation of the *psbC*(WT)-*aadA* mRNA or the *psbC* mRNA, as demonstrated by RNA blot analysis of total RNA samples from a representative tetrad from each cross (Fig. 1 C). However, there is an enhanced accumulation of the *psbC-aadA* transcript in the progeny bearing the *tbc2-G314* allele (Fig. 1 C, lanes 3 and 4) and the *tbc2-B23* allele (Fig. 1 C, lane 7). Because the promoter and 5'UTRs are the same for the *psbC* and *psbC-aadA* RNAs, these differences are due to the different coding sequences and 3'UTRs. In contrast, the *aadA* protein was not produced from the *psbC* 5'UTR in any of the PSII deficient progeny; all were sensitive to spectinomycin (Fig. 1 D). The wild-type (photosynthetic) progeny expressed *aadA*; all were resistant to the drug (Fig. 1 D). Therefore, the new mutations in *G314* and *B23* eliminate the expression of *aadA* from the *psbC* 5'UTR. As these effects occur in the absence of the P6 coding sequence and are mediated by the 5'UTR, a sequence element that is involved in translational regulation of *psbC* (Zerges and Rochaix, 1994; Zerges et al., 1997), we conclude that both mutations abolish translation of the *psbC* mRNA. Therefore, *G314* and *B23* identify new mutations that specifically abolish translation of the *psbC* mRNA.

To determine whether these new *TBC* mutations are in either of the two known *TBC* loci, we asked whether they are allelic to *tbc1-F34* or *tbc2-F64*. *G314* and *B23* were crossed to strains carrying *tbc1-F34* or *tbc2-F64*, and the resulting zygotes were tested for photosynthetic activity by fluorescence transient analysis (Bennoun et al., 1980). As all of the parental strains are completely PSII deficient, the appearance of PSII

Table I. Genetic analysis of *tbc2* mutants

	<i>tbc2-F64</i>	<i>tbc2-G314</i>	<i>tbc2-b23</i>	<i>tbc1-F34</i>
<i>tbc2-F64</i>	$\leq 10^{-6}$	—	—	+
<i>tbc2-G314</i>	0/62	$\leq 410^{-6}$	—	+
<i>tbc2-b23</i>	0/20	0/41	$\leq 10^{-9}$	+
<i>tbc1-F34</i>	42/45	20/30	18/32	$\leq 510^{-6}$

Results of the complementation test are shown above the diagonal. The young zygotes displayed either (PSII) (–) or wild-type phenotype (+). Results of the recombination tests are shown below the diagonal. The scores represent a1/a2, where a1 is the number of zygotes that germinated and gave rise to colonies on minimal medium, and a2 is the number of zygotes that germinated. Frequencies of spontaneous reversion of mutations are shown in diagonal (109 cells tested on minimal medium). The results of the same tests performed between the *tbc2* mutants and the *tbc1-F34* mutant strain are shown as controls.

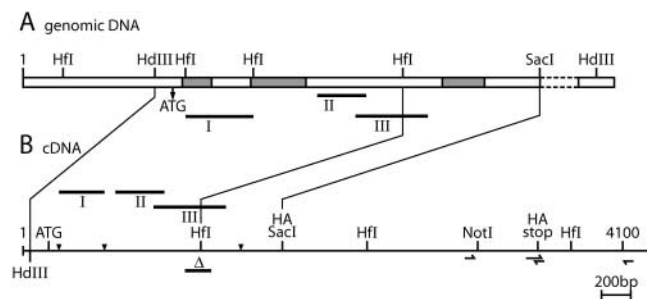
activity in the zygotes would indicate complementation of the mutations being tested; i.e., that the respective mutations are nonallelic and, therefore, are probably in distinct genes. The zygotes obtained from the crosses of *G314* or *B23* to a strain carrying *tbc1-F34* gave wild-type fluorescence transients, indicating that neither of the new mutations is allelic to *tbc1-F34* (Table I). In addition, the recombination tests from the crosses of *G314* and *B23* to the strain carrying *tbc2-F64* did not yield any photosynthetic progeny, indicating that the mutant alleles in these strains are linked (Table I). In contrast, crosses of *G314* and *B23* to the strain carrying *tbc1-F34*, which is not linked to *TBC2*, yielded many photosynthetic progeny (Table I). Therefore, the mutations in *G314* and *B23* are allelic and linked to *tbc2-F64*, and we have named them *tbc2-G314* and *tbc2-B23*, respectively. The positive complementation between the three *tbc2* alleles and *tbc1-F34* indicate that all three *tbc2* mutant alleles are recessive.

### Complementation and cloning of *TBC2* and its cDNA

In order to clone the *Tbc2* gene, pools of genomic cosmid clones from an indexed cosmid library (Zhang et al., 1994) were tested for their ability to complement the photosynthesis deficiency produced by *tbc2-B23*. One pool yielded five photoautotrophic colonies. From this pool we isolated a cosmid that complements all three mutant *tbc2* alleles (see Materials and methods).

To subclone the rescuing cosmid DNA, restriction fragments obtained from single, double, and triple digests of the cosmid DNA were used to transform the three *tbc2* mutant strains. A *Bam* HI fragment of  $\sim 9.5$  kb was identified that was able to complement all three alleles. No subfragments of this *Bam* HI fragment were found to be able to complement the mutant phenotypes, suggesting that the DNA encodes a functional *TBC2* gene with little extraneous sequence. This *Bam* HI fragment was cloned and partially sequenced. It was found to contain two *Hind* III sites and, upon digestion with *Hind* III, fragments of  $\sim 0.9$ , 3.8, and 4.8 kb were generated. The two larger fragments were used to screen  $6 \times 10^5$  phage of a cDNA library. Initially, only one 4.1-kb cDNA was isolated, and it hybridized to both fragments. However, this cDNA was unable to rescue the mutants. It was cloned and sequenced in its entirety. Analysis of its coding capacity (GeneMark 2.4) indicated a probable frame shift around residue 1200. Indeed, fusions between the 5' genomic DNA (up to



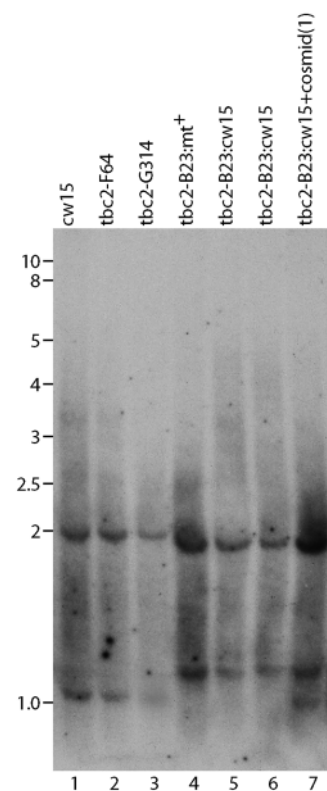


**Figure 2. Map of the *TBC2* locus and its cDNA.** (A) Region of the promoter region and 5' part of the *Tbc2* gene. The first three introns are indicated in grey. *HinfI* sites (Hfi, at 273; 1,110; 1,574; and 2,596 bp), and the approximate location of the *HindIII* sites used to generate probes to clone the cDNA are shown (HdIII). The positions of probes I (1110–1574), II (2011–2346), and III (2273–2767) on the genomic DNA are indicated. Dotted regions indicate unsequenced DNA (this section is not to scale). (B) The map of the longest *Tbc2* cDNA (4,098 nucleotides without the polyA tail) is represented. The unique *SacI* (1771) and *NotI* (3108) sites as well as the start codon (ATG, 174–176) and stop codon (stop, 3519–3521) are also noted. The location of probes I (195–506), II (630–965), and III (892–1386) relative to the cDNA sequence are shown. The  $\Delta$  indicates the region deleted in three from eight cDNAs examined (1107–1285). The PCR primers used to insert a *HincII* site immediately upstream of the stop codon are indicated by arrows. The HA epitope was inserted into this *HincII* site or at the *SacI* site. The locations of the first three introns are marked by wedges. The corresponding *HindIII*, *HinfI*, and *SacI* sites on the genomic and cDNA are shown by thin lines.

the *SacI* site) and the cDNA downstream of the *SacI* site were able to complement the *TBC2* mutations, indicating that the 3' half of the cDNA (downstream of the putative frameshift) encodes a functional protein (Fig. 2; unpublished data). Screening another  $8 \times 10^5$  phage of the cDNA library yielded eight additional phage, two of which were able to complement. The 5' halves of the rescuing cDNAs were sequenced and found to contain an additional internal 179-bp DNA fragment relative to the first cDNA, also found in the genomic sequence, that restored the reading frame (nucleotides 1107–1285 of the cDNA). PCR analysis was performed on the other cDNAs unable to rescue the mutants. Three contain a similar deletion to that of the first cDNA, one is missing a different internal region, and the other two do not have any detectable rearrangements (unpublished data). It is not yet known whether the differences observed are due to alternative splicing or to cloning artifacts.

### Molecular analysis of *TBC2*

To confirm that the cloned gene corresponds to *TBC2*, we examined restriction digestion patterns within the three alleles (Fig. 3). Genomic DNA was digested with *HinfI*, blotted, and hybridized to different probes. Using a PCR-amplified cDNA fragment corresponding to nucleotides 892 to 1386 (probe III), two *HinfI* fragments of 2.0 and 1.0 kb were detected in the wild-type, *tbc2-F64*, and *tbc2-G314*. However, the same probe detected a 1.9- and a 1.2-kb fragment in *tbc2-B23*, suggesting that this was an allele-specific restriction site difference. When the genomic DNA from a *tbc2-B23* strain that had been rescued using the whole cosmid clone was hybridized to the same probe, four fragments were detected: the 2.0- and 1.0-kb frag-



**Figure 3. DNA blot analysis of the *TBC2* locus in wild-type and the *tbc2* mutants.** 5  $\mu$ g of the indicated genomic DNA was digested with *HinfI*, separated on a 0.7% agarose gel, transferred to Hybond-C extra, and probed with PCR-amplified probe III (Fig. 2). Size markers are indicated in kb on the left of the gel (Eurogentech Smart ladder). The 2-kb fragment in lane 7 is undetectable because of overexposure.

ments from the wild-type *Tbc2* gene, and the 1.9- and 1.2-kb fragments specific to *tbc2-B23* (lane 7). Hybridization of the same blot with probe II (Fig. 2, a *PstI* fragment corresponding to cDNA nucleotides 630–960) detected the 1.2-kb fragment in *tbc2-B23*, and the 1.0-kb fragment in the other allelic mutants (unpublished data). Hybridization with probe I (a genomic *HinfI* fragment) corresponding to the 466-bp genomic fragment (Fig. 2) revealed no differences between the wild-type, *tbc2-B23*, and *tbc2-G314*. Thus, a DNA rearrangement has occurred near the *HinfI* site comprised within probe III. Taken together, these results strongly suggest that this DNA rearrangement in *tbc2-B23* is the cause of the mutations in these alleles.

To test whether the *TBC2* locus is linked to other nuclear loci involved in PSII biogenesis, the *tbc2-F64* mutant was crossed with two PSII-deficient strains, *ac-114* and *ac-115*, containing mutations that have been mapped on linkage groups III and I, respectively (Harris, 1989). Analysis of the progeny from 20 tetrads of the cross with *ac-114*, and 8 tetrads of the cross with *ac-115*, indicated that the *TBC2* locus is not linked to these two loci (see Materials and methods).

### Characterization of the predicted *TBC2* amino acid sequence

The *Tbc2* cDNA sequence predicts an ORF encoding a protein of 1,115 amino acids with a molecular mass of 114.8 kD (Fig. 4). The longest isolated cDNA has a 5' UTR of 174





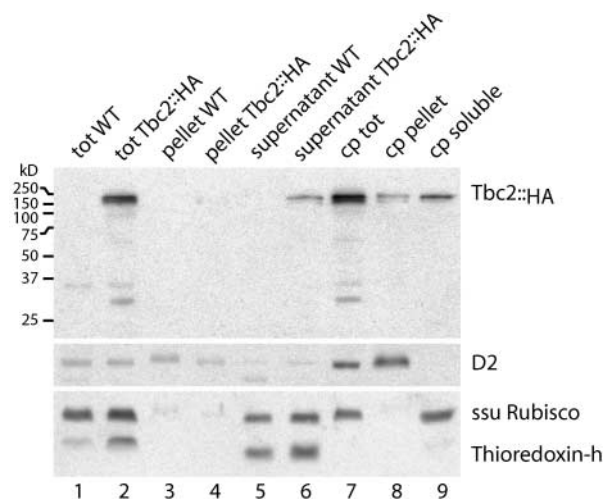
clear gene and has been implicated in the processing and translation of the chloroplast *petA* and *petD* RNAs and contains PPR repeats (A. Barkan, personal communication; Small and Peeters, 2000). As can be seen in Fig. 4 A, the sequence similarity between Crp1 and Tbc2 extends over the central region of Tbc2 that contains the PPPEW repeats. Fig. 4 D shows that this region comprises the first seven PPPEW repeats of Tbc2 and the first seven PPR repeats of Crp1. In spite of this overlap, the PPR repeats of Crp1 are distinct from the PPPEW repeats of Tbc2, although they share three consensus residues, L, G, and P in the COOH-terminal part of the repeats (Fig. 4 D). However, the PPPEW motif is not present in the PPR repeats and the NH<sub>2</sub>-terminal parts of the two repeat families are unrelated. Secondary structure predictions indicate considerable amounts of  $\alpha$ -helical structures in Tbc2 (PSIPRED, <http://insulin.brunel.ac.uk/psipred/>), but no evident folds. In addition, no known RNA-binding motifs can be detected.

To further characterize the Tbc2 protein, its gene was tagged with a triple hemagglutinin (HA) epitope in either the middle of the coding sequence, at codon 593, or immediately before the stop codon (Fig. 4 A). The constructs were used separately for transformation of a *C. reinhardtii* strain carrying the *tbc2-F64* mutation by selecting for growth on minimal medium. Several independent colonies from each transformation were screened by immunoblotting for expression of the HA epitope-tagged Tbc2 protein. Although both constructs were able to rescue the mutant and expressed detectable HA-tagged protein of the same size, and are therefore functional, the signal from the construct with the epitope at the COOH terminus yielded a much stronger signal (unpublished data). It was used exclusively in the experiments that follow.

### Characterization of the Tbc2 protein

The NH<sub>2</sub> terminus of Tbc2 is enriched in basic and hydroxylated amino acids, but also contains a higher portion of acidic residues than expected for a typical chloroplast transit peptide. Analysis of this sequence with PSORT (<http://psort.nibb.ac.jp/>) or ChloroP (<http://www.cbs.dtu.dk/services/ChloroP/>) predicts a mitochondrial rather than a chloroplast location. However, a particular feature of chloroplast transit peptides from *C. reinhardtii* is that they share features with both chloroplast and mitochondrial target sequences of vascular plants (Franzen et al., 1990).

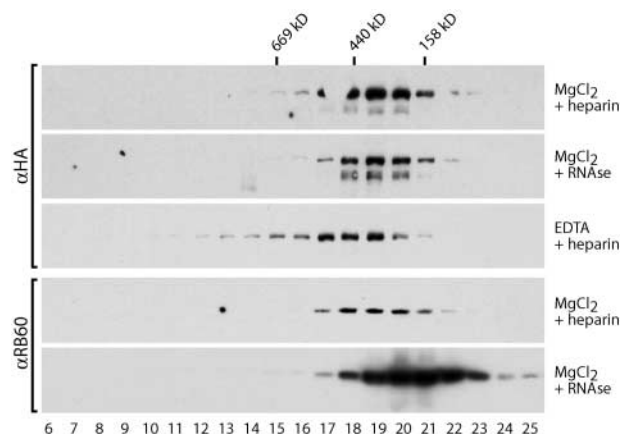
In order to determine the subcellular location of Tbc2 directly, cells from the mutant *tbc2-F64* were transformed with the HA epitope-tagged *Tbc2* construct by selecting for photoautotrophic growth. Cells were gently broken and chloroplasts were isolated by centrifugation on Percoll gradients and fractionated into a soluble and membrane fraction by high-speed centrifugation. The proteins were then separated on 8% polyacrylamide gels and probed with antisera raised against several proteins (Fig. 5). The monoclonal HA antibody detected a protein of ~140 kD in extracts from whole cells (lane 2), chloroplasts (lane 7), and the chloroplast-derived soluble fraction (lane 9), but considerably less in the chloroplast membranes (lane 8). In other independent extractions, the signal in the chloroplast membrane fraction was undetectable, especially when sonication of the chloro-



**Figure 5. Accumulation and subcellular localization of Tbc2.** Immunoblot analysis of Tbc2 in wild-type (lanes 1, 3, and 5) and *tbc2-F64* rescued with the HA-tagged *Tbc2* cDNA (lanes 2, 4, and 6–9); tot, total cell extract (lanes 1, 2); total insoluble fraction (lanes 3 and 4); total soluble fraction (lanes 5, 6); cp total, total chloroplast extract (lane 7), chloroplast insoluble fraction (lane 8), chloroplast soluble fraction (lane 9). After fractionation by SDS-PAGE and blotting, the immunoblots were reacted with antisera raised against HA, D2, SSU Rubisco, and thioredoxin-h (cytosolic protein). For details see Materials and methods.

plasts was omitted. The protein detected migrates as if it were ~20 kD larger than expected (including 4.2 kD for the triple HA epitope), and is not detected in either nonrescued cells (lanes 1, 3, and 5) nor in cells rescued with a non-HA-bearing *Tbc2* gene (unpublished data). The protein is found in chloroplasts, and is detected mostly in the soluble fraction (Fig. 5, lane 9). In the experiment presented in Fig. 5, the chloroplast extracts were not treated with salt. However, a 0.5-M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> wash to remove loosely bound proteins from the membranes before centrifugation (Zerges and Rochaix, 1998) did not alter the fractionation results obtained with Tbc2 (unpublished data). As a control for the fractionation experiment, the distribution of a soluble chloroplast protein (ribulose-bis-phosphate carboxylase-oxygenase; Rubisco), D2, a PSII reaction center subunit in the thylakoid membrane, and thioredoxin-h, a cytosolic marker protein, were also determined (Fig. 5). Each of these proteins fractionated as expected, indicating that the chloroplasts used were not significantly contaminated with cytosolic proteins. Thus, despite the absence of an identifiable chloroplast transit peptide, the Tbc2 protein is targeted to the chloroplast. Similar results were obtained using cells complemented with Tbc2 HA-epitope tagged in the middle of the protein (in the *Sac* I site, Fig. 2 B; unpublished data). This substantiates the chloroplast localization of the protein.

We expected that Tbc2 might form a complex with other proteins and possibly also with RNA in order to exercise its function in *psbC* mRNA translation. To test this possibility, we fractionated total soluble cell extracts by size exclusion chromatography (Fig. 6). The column used fractionates proteins and complexes over the size range of 5–2,000 kD. In the presence of 10 mM MgCl<sub>2</sub>, 50 mM KCl and 1 mg/ml



**Figure 6. The Tbc2 protein is part of a high molecular weight complex.** A total soluble cell extract from a strain carrying the *tbc2-F64* allele and expressing the HA-tagged *Tbc2* cDNA was concentrated to a final concentration of 15–20  $\mu\text{g}/\text{ml}$  and fractionated on a Superose 6 PC3.2/30 column using the SMART system (Amersham Pharmacia Biotech). Fractionation was performed in the presence of  $\text{MgCl}_2$  and heparin (rows 1 and 4),  $\text{MgCl}_2$  and RNase (rows 2 and 4), or EDTA and heparin (row 3). 25 fractions were collected and aliquots were electrophoresed on an 8% SDS-polyacrylamide gel and reacted with anti-HA monoclonal antibody (rows 1–3) and antibodies against RB60 (rows 4 and 5).

heparin, Tbc2 elutes as a complex with a peak at  $\sim 400$  kD (fraction 19). Very little signal is detected in fraction 22, where a monomeric form of the protein would be expected to elute. Treatment of the whole cell lysate with RNase does not alter the migration of the complex on the column, indicating that the protein is probably not stably associated with RNA ( $\alpha$ -HA, middle). As a control, the same filters were reprobed with an antibody against RB60, which is known to be part of an RNA–protein complex (Danon and Mayfield, 1991; Boudreau et al., 2000). In untreated samples the Tbc2:HA and RB60 proteins fractionate in a similar fashion. However, upon RNase treatment, the RB60 complex shifts to a lower molecular weight, indicating that Tbc2 and RB60 are not part of the same complex. The Tbc2 protein does not elute in the same fractions as Hsp70 and is thus not associated with this protein (unpublished data). When the whole-cell extract was prepared in the presence of 50 mM KCl, 10 mM EDTA and 1 mg/ml heparin, but without  $\text{MgCl}_2$ , the Tbc2 complex was observed to have a higher apparent molecular weight and was more spread out over the column. This may be indicative of some aggregation in the absence of divalent cations.

Because Tbc2 is involved in translation of *psbC* mRNA we tested whether it is associated with polysomes. Polysomes were isolated from whole-cell extracts as rapidly as possible to prevent RNA degradation by centrifugation using two-step sucrose cushions. Immunoblot analysis of the supernatant and the polysome-containing pellet indicated that Tbc2:HA remains in the supernatant, and thus is not stably associated with polysomes (unpublished data). Also, if Tbc2 were associated with polysomes, one would expect a lower molecular mass without  $\text{Mg}^{2+}$ , not a higher mass as was observed.

## Discussion

Genetic analyses of mutants of *C. reinhardtii* deficient in photosynthetic activity have revealed several nuclear loci required for the expression of the chloroplast *psbC* gene. One nuclear locus is required for the stable accumulation of *psbC* mRNA (Sieburth et al., 1991) and at least three loci, *TBC1*, *TBC2*, and *TBC3* are involved in the translation of this mRNA, most likely at the level of initiation (Zerges and Rochaix, 1994; Zerges et al., 1997). This study represents a first step toward the identification and characterization of the transacting factors specified by these loci, in this case the *TBC2* locus. Because the *tbc2-F64* mutation undergoes phenotypic reversion at a frequency that is too high to select for complementation by pools of cosmids from the indexed library (see Materials and methods), we had to isolate more stable mutant alleles of the *TBC2* locus. One of these, *tbc2-B23*, was identified and used for the cloning of the *Tbc2* gene through genomic complementation with an indexed cosmid library.

Other loci involved in PSII biogenesis have been mapped previously. They include the *AC-114* locus on linkage group III and *AC-115* on linkage group I. Analysis of the progeny from crosses between the *tbc2-F64* mutant and the *ac-114* and *ac-115* mutants revealed that the *TBC2* locus is unlinked to these two loci. Thus, there is no evidence for the clustering of nuclear genes involved in posttranscriptional steps of the expression of plastid genes encoding PSII subunits.

### Structural features of Tbc2

The *Tbc2* gene encodes a protein of 1,115 amino acids. A noticeable feature of this protein is that it contains nine degenerate repeats of 38–40 amino acids that extend from the middle to the COOH-terminal region. Although the Tbc2 protein is not obviously related to any protein in the data bases, it is particularly interesting that the maize Crp1 protein is found among the proteins that displays low sequence identity with Tbc2. Crp1 is required for the processing and translation of the plastid *petA* and *petD* RNAs (Fisk et al., 1999). Crp1 contains PPR repeats (Small and Peeters, 2000) that are similar to TPR repeats, except that the repeat consists of 35 residues. The region of sequence similarity between Tbc2 and Crp1 includes the repeated motifs of these two proteins, although the PPR motifs are distinct from the internal repeats of Tbc2 aside from sharing three consensus residues in their COOH termini. The presence of TPR motifs has been found in a large set of proteins involved in many different activities such as cell cycle control, transcription, protein import into mitochondria, and chloroplast RNA metabolism (Blatch and Lassel, 1999). In particular, the Nac2 and Mbb1 proteins containing 9 to 10 TPR-like repeats are required for the stable accumulation of the chloroplast *psbD* and *psbB* mRNAs in *C. reinhardtii* (Boudreau et al., 2000; Vaistij et al., 2000). Changing one conserved residue of one of the Nac2 TPR repeats abolishes the activity of the protein, indicating that these repeats have an important functional role (Boudreau et al., 2000).

All of these proteins appear to belong to a large family of helical repeat proteins. The atomic structure of several representatives of these proteins has been determined. They in-

clude  $\beta$  catenin with its 12 Arm repeats of 42 amino acids (Huber et al., 1997), the A subunit of protein phosphatase 2A with its 15 HEAT repeats of 39 amino acids (Groves et al., 1999), Pumilio with its 8 Puf repeats of 36 amino acids (Edwards et al., 2001), and protein phosphatase 5 with its 3 TPR repeats of 34 amino acids (Das et al., 1998). These tandem helical repeats form an extended surface of the protein that is thought to be involved in protein–protein interactions. In the case of Pumilio, a translational regulator of the *hunchback* mRNA in *Drosophila*, this surface can also be used for recognizing RNA (Edwards et al., 2001). Although Tbc2 does not contain any known repeat, it is predicted to contain a considerable amount of  $\alpha$ -helical structures and a novel repeated sequence. It is not yet known whether or not Tbc2 has RNA binding activity.

Further searches for sequence similarity revealed that Tbc2 shares a short stretch of 42 amino acids with selenophosphate synthase (52% sequence identity). Other unusual structural features of Tbc2 include the presence of long stretches of Ser, Ala, or Gln mostly in the NH<sub>2</sub>- and COOH-terminal regions that have also been noticed in other nucleus-encoded proteins from *C. reinhardtii* (Boudreau et al., 2000; Vaistij et al., 2000).

### Tbc2 is a chloroplast stromal protein

The NH<sub>2</sub>-terminal region of Tbc2 does not resemble typical chloroplast transit peptides but is recognized as a mitochondrial presequence using PSORT or ChloroP subcellular localization programs. This finding is not necessarily incompatible with a chloroplast location, because the analysis of chloroplast transit peptides from *C. reinhardtii* has revealed that they share features with both mitochondrial and higher plant chloroplast presequences (Franzen et al., 1990). They contain both the potential amphiphilic  $\alpha$ -helix of mitochondrial presequences and the amphiphilic  $\beta$ -strand of higher plant chloroplast transit peptides. It is also possible that the chloroplast targeting sequence is located elsewhere in the protein.

To determine the location of Tbc2, the protein was tagged with an HA epitope and was localized mainly in the stromal compartment of the chloroplast by cell fractionation and immunoblotting. It is interesting to note that among the nucleus-encoded factors known to be involved in chloroplast post-transcriptional steps, some, like Tbc2, Crp1 (Fisk et al., 1999), Nac2 (Boudreau et al., 2000), Mbb1 (Vaistij et al., 2000), and Raa3 (Rivier et al., 2001), are found in the soluble chloroplast phase, whereas others, like RB47 and Raa2, are associated with a low-density membrane system (Zerges and Rochaix, 1998; Perron et al., 1999). RB60 appears to be partitioning both with the stroma (Boudreau et al., 2000) and thylakoids (Trebitsh et al., 2001). The functional significance of this different compartmentalization remains to be determined. It is possible that several of these factors interact only transiently with the chloroplast membrane during some of the steps leading to the integration of the newly synthesized polypeptides into the membranes. Although the HA-tagged Tbc2 protein is functional and rescues the *tbc2* mutations, we cannot exclude that it may not behave exactly the same way as the authentic Tbc2 protein.

Tbc2 appears to be part of a high molecular weight complex of 400 kD. It could represent a homomeric complex, or

Tbc2 could be associated with other factors. Possible candidates include Tbc1 and Tbc3, which are known to be involved in the initiation of translation of the *psbC* mRNA (Zerges et al., 1997). The Tbc2 complex does not appear to contain RNA based on the observation that its size is not altered by RNase treatment. In addition, as for Crp1, no stable association of Tbc2 with polysomes could be detected, suggesting that this factor interacts only transiently or indirectly with the translational machinery. As Tbc2 has a specific role in the translation of the *psbC* mRNA and its action is mediated through the *psbC* 5'UTR, it has to functionally interact with this region. Whether this interaction occurs directly or indirectly through other RNA-binding factors remains to be determined. Most factors involved in translation have been identified by biochemical approaches. This study, and others described here, reveal that genetic approaches can identify new translational regulators. The culmination of these approaches will be a comprehensive understanding of the molecular mechanisms underlying translation in vivo.

## Materials and methods

### *C. reinhardtii* strains

*C. reinhardtii* strains (wild-type 137c) were grown on TAP medium (Gorman and Levine, 1966), an enriched medium that allows for mixotrophic growth, under 50  $\mu\text{E}/\text{m}^2/\text{s}$  light unless otherwise indicated. Cells were grown on minimal medium (HSM; Harris, 1989) to select for photosynthetic growth. *G314* and *B23* were isolated from the wild-type strain 137c after treatment with 5-fluorodeoxyuridine as described (Bennoun et al., 1986), and a subsequent enrichment for photosynthetic mutants with metronidazole as described (Schmidt et al., 1977).

### Genetic analysis

Mating, germination, and tetrad analysis were performed according to Harris (1989). Reversion and recombination tests were performed as described (Kuras et al., 1997), and complementation tests were done according to Goldschmidt-Clermont et al. (1990).

### DNA isolation

Total *Chlamydomonas* DNA was prepared as described (Boudreau et al., 1997). Gels were transferred to Hybond N+ or Hybond C-extra (Amersham Pharmacia Biotech), and hybridized and washed as described (Church and Gilbert, 1984). PCRs were performed with a 5-ng/ $\mu\text{l}$  template and 0.5- $\mu\text{M}$  primers in 50 mM Tris-Cl, pH 8.3, 1 mM MgCl<sub>2</sub>, 250  $\mu\text{g}/\text{ml}$  BSA, 200  $\mu\text{M}$  dNTP, and 5% DMSO using *Pfu* polymerase (Stratagene).

### Pulse labeling of proteins

Pulse labeling of cells with [<sup>14</sup>C]acetate was performed as described (Rochaix et al., 1989). At the end of the of the labeling period, 200 ml of culture was used for thylakoid membrane purification, and the extract was fractionated by electrophoresis on 7.5–15% SDS polyacrylamide gels (Chua and Bennoun, 1975).

### Complementation and cloning of the *Tbc2* gene and cDNA

An indexed cosmid library (Zhang et al., 1994) of *C. reinhardtii* genomic DNA was used to transform *tbc2-B23;cw15* essentially as described (Kindle, 1990). 2.5  $\mu\text{g}$  of pooled DNA from each microtiter plate was used to transform  $3 \times 10^7$  cells in the presence of 100  $\mu\text{l}$  20% PEG 8000 (in 5 mM Tris-Cl, pH 8.0) using sterile 0.4-mm glass beads (Thomas Scientific). Cells were plated on high-salt minimal medium and immediately placed under medium intensity light (60  $\mu\text{E}/\text{m}^2/\text{s}$ ). Positive plates were generally detected after 14–18 d. Library plate number 64 yielded 0–2 colonies per plate after transformation. DNA was prepared from each column and row to identify the well containing the cosmid that complements the *tbc2* mutation (F5). 1  $\mu\text{g}$  of purified cosmid DNA yielded six colonies in *tbc2-B2;cw15* (no DNA control yielded 0 colonies); 93 transformants in *tbc2-F64;cw15* (no DNA control yielded 19 colonies); and 315 colonies in *tbc2-G314;cw15* (no DNA control yielded two colonies per plate).

To identify which region of the cosmid rescued the *tbc2* mutants, the cosmid DNA was digested with different restriction enzymes. The smallest



fragment that was able to complement was a 9.5-kb *Bam*HI fragment. This fragment was cloned into pBluescriptKS- (Stratagene) in which the *Sac*I, *Sal*I, *Hind*III, and *Not*I polylinker sites had been deleted, and was partially sequenced. This *Bam*HI fragment contains two *Hind*III sites (Fig. 2). The two largest *Hind*III fragments (~4.8 and 3.8 kb in size) were used separately to screen  $6 \times 10^5$  phage of a cDNA library constructed by H. Sommer (Max Planck Institute für Züchtungsforschung, Cologne, Germany). One cDNA that hybridized to both fragments was sequenced in its entirety by making exonucleaseIII deletions of the cDNA (Sambrook et al., 1989). A subsequent round of screening of  $8 \times 10^5$  phage using a 5'-terminal region of the cDNA (nucleotides 893–1208 of the first cDNA) identified another eight cDNA clones. The 5'-terminal ends of two were sequenced. The sequences were assembled using AssemblyLIGN software (Oxford Molecular Ltd).

### Epitope tagging of Tbc2

The triple HA epitope was inserted into the *Sac*I site in the middle of the genomic coding sequence of *Tbc2* (Fig. 2). The triple HA epitope was also inserted at the COOH-terminal end of the protein. A *Sal*I/*Hinc*II site was first introduced upstream of the stop codon of the genomic *Tbc2* DNA to allow for the insertion of the tag. The HA epitope-containing DNAs were introduced into *tbc2-F64;cw15* by selecting for restoration of photosynthesis, and the transformants were screened for expression of the HA epitope by immunoblotting. HA epitope-tagged strains were maintained on HSM to ensure continued expression of the tagged protein.

### Immunoblotting and cell fractionation

Total protein extracts obtained from cell pellets of 10-ml cultures were resuspended in 250  $\mu$ l HKM (Hepes-KOH 20 mM, pH 7.8, KCl 50 mM, MgCl<sub>2</sub> 10 mM) containing protease inhibitors: 5 mM  $\epsilon$ -amino caproic acid, 1 mM benzamide HCl, 25  $\mu$ g/ml pepstatin A, and 10  $\mu$ g/ml leupeptin, and then sonicated on ice. A portion of this extract was centrifuged at 100,000 *g* for 30 min at 4°C, giving rise to a pellet and supernatant fraction. The pellet was washed with 1 ml of STN buffer (0.4 M sucrose, 100 mM Tris, pH 8.0, 10 mM NaCl) and centrifuged again for 15 min at 50,000 *g* at 4°C to remove soluble contaminating proteins. The pellet was resuspended in lysis buffer (50 mM Tris, pH 6.8, 2% SDS, 10 mM EDTA, and protease inhibitors), incubated for 1 h at room temperature, and centrifuged for 5 min in a microfuge. The resulting supernatant was used as total insoluble protein extract.

Chloroplasts were isolated from a strain carrying the *tbc2-F64* and *cw15* alleles, which had been transformed with *Tbc2:HA*. A 500 ml culture of cells grown to mid-log phase was centrifuged and resuspended in 10 ml of breaking buffer (300 mM sorbitol, 50 mM Hepes-KOH, pH 7.8, 5 mM MgCl<sub>2</sub>) with protease inhibitors. Saponin (S-4521; Sigma-Aldrich) was added to a final concentration of 0.25% (from a freshly made solution of 10% saponin in H<sub>2</sub>O), incubated for 1 min on ice, and then centrifuged for 3 min at 3,000 rpm (Sorvall, HB6) at 4°C. The pellet containing intact chloroplasts was resuspended carefully in 10 ml of breaking buffer with protease inhibitors, and loaded on a discontinuous 45–75% Percoll gradient prepared in breaking buffer with protease inhibitors as described (Zerges and Rochaix, 1998). Chloroplasts were collected at the 45–75% interface, washed once in breaking buffer, and osmotically lysed in chloroplast lysis buffer (10 mM Tricine, 5 mM MgCl<sub>2</sub>, 2 mM DTT, and protease inhibitors). The soluble and insoluble fractions were prepared as described above. 50  $\mu$ g of protein were loaded on a 10–18% linear gradient SDS-PAGE gel and electroblotted to nitrocellulose membranes, and reacted with antisera against the following proteins or epitopes: HA, D2, SSU Rubisco, and cytosolic thioredoxin-h.

Alternatively, chloroplasts were isolated from *tbc2-F64;cw15* cells transformed with HA epitope-tagged Tbc2 essentially as described (Zerges and Rochaix, 1998) from continuous 10–80% Percoll gradients. Intact chloroplasts were collected, diluted in 0.3 M sorbitol, 50 mM Hepes-KOH, pH 7.8, and 5 mM MgCl<sub>2</sub>, and centrifuged for 5 min at 4°C. After adding protease inhibitors, the chloroplasts were broken by repeated pipetting in 50 mM Hepes-KOH, pH 7.8, and 5 mM MgCl<sub>2</sub>, and frozen at –80°C. The lysate was thawed on ice, KCl was added to 50 mM, MgCl<sub>2</sub> to 10 mM, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 0.5 M, and was left to precipitate on ice for 45 min. The lysate was centrifuged at 100,000 *g* for 30 min to yield a membrane pellet, and a soluble chloroplast extract that was concentrated in a Centricon C-30 (Amicon). 8% gels were loaded with 50  $\mu$ g protein (to detect Tbc2:HA and eIF4a) or with 10  $\mu$ g protein (to detect Rubisco and Psal).

Whole-cell extracts were used for size fractionation. Cells were grown and harvested as above and resuspended in either HMK (20 mM Hepes-KOH, pH 7.8, 50 mM KCl, 10 mM MgCl<sub>2</sub>) or HEK (20 mM Hepes-KOH, pH 7.8, 50 mM KCl, 10 mM EDTA) plus protease inhibitors (as above) in

the presence or absence of 1 mg/ml heparin. Cells were broken by passing once through a small French Press cell (SLM-Aminco; Spectronic Instruments) at 1,380 lb/in<sup>2</sup>. RNase was added to 10  $\mu$ g/ml when necessary, and incubated for 10 min at room temperature. The lysate was centrifuged at 35,000 *g* for 45 min, then at 100,000 *g* for another 45 min to yield a soluble extract that was concentrated to 15–20 mg/ml protein. An aliquot of 50  $\mu$ l was loaded onto a Superose 6 PC 3.2/30 column using the SMART system (Amersham Pharmacia Biotech), run with either HMK or HEK at 40  $\mu$ l/min, and 1–1.2 MPa at 4°C. 50- $\mu$ l fractions were collected, of which 20  $\mu$ l were loaded on an 8% gel. The molecular weight range of the column was estimated using the MW-GF-1000 size standard kit (Sigma-Aldrich). Polysomes were prepared as described (Boudreau et al., 2000).

Protein samples were incubated with an equal volume of 80 mM Tris-Cl, pH 6.8, 1.6% SDS, 80 mM DTT, 30% glycerol, 8 mM  $\epsilon$ -NH<sub>2</sub> caproic acid, 1.6 mM benzamide, 1.6  $\mu$ g/ml leupeptin, 1.6  $\mu$ M E64, 32  $\mu$ g/ml pepstatin A, 2.6  $\mu$ M PMSF, 1.6  $\mu$ M 1,10 *o*-phenanthroline, and a 1:100 dilution of Sigma protease inhibitors P 8849 for 20 min at ambient temperature before loading on polyacrylamide gels. Gels were transferred to Protran nitrocellulose membranes (Schleicher and Schuell), blocked in a Tris-buffered salt solution containing 0.1% Triton X-100 and 4% nonfat milk for 1 h, and incubated with primary antibody overnight at 4°C. Primary antisera used were a mouse  $\alpha$ -HA (1:1,000, Eurogentec),  $\alpha$ -thioredoxin-h (1:1,000),  $\alpha$ -Rubisco (1:5,000),  $\alpha$ -PsbD (D2) (1:10,000), and  $\alpha$ -RB60 is rabbit (1:1,000). Horseradish peroxidase-coupled secondary antibody at 1:10,000 was used to detect binding by chemiluminescence (Durrant, 1990).

We thank C. Steinberger for her help in detecting and localizing the PPPEW repeats, A. Barkan for communicating unpublished results, L. Falquet for his help with similarity searches, M. Goldschmidt-Clermont for helpful comments, A. Danon (Weizman Institute of Science, Rehovot, Israel) for RB60 antibodies, H. Sommer for constructing the cDNA library, E. Boudreau, S. Lemaire, and C. Rivier for valuable advice and opinions, M. Rahire for help with cDNA sequencing, and N. Roggli for preparing the figures.

This work was supported by grant 3100-050895.97 from the Swiss National Fund.

Submitted: 15 January 2002

Revised: 30 April 2002

Accepted: 30 April 2002

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