

# Tab2 is a novel conserved RNA binding protein required for translation of the chloroplast *psaB* mRNA

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**The chloroplast *psaB* mRNA encodes one of the reaction centre polypeptides of photosystem I. Protein pulse-labelling profiles indicate that the mutant strain of *Chlamydomonas reinhardtii*, *F14*, affected at the nuclear locus *TAB2*, is deficient in the translation of *psaB* mRNA and thus deficient in photosystem I activity. Genetic studies reveal that the target site for Tab2 is situated within the *psaB* 5'UTR. We have used genomic complementation to isolate the nuclear *Tab2* gene. The deduced amino acid sequence of Tab2 (358 residues) displays 31–46% sequence identity with several orthologues found only in eukaryotic and prokaryotic organisms performing oxygenic photosynthesis. Directed mutagenesis indicates the importance of a highly conserved C-terminal tripeptide in Tab2 for normal *psaB* translation. The Tab2 protein is localized in the chloroplast stroma where it is associated with a high molecular mass protein complex containing the *psaB* mRNA. Gel mobility shift assays reveal a direct and specific interaction between Tab2 and the *psaB* 5'UTR. We propose that Tab2 plays a key role in the initial steps of PsaB translation and photosystem I assembly.**

**Keywords:** *Chlamydomonas*/chloroplast/photosynthesis/photosystem I mutant/translation

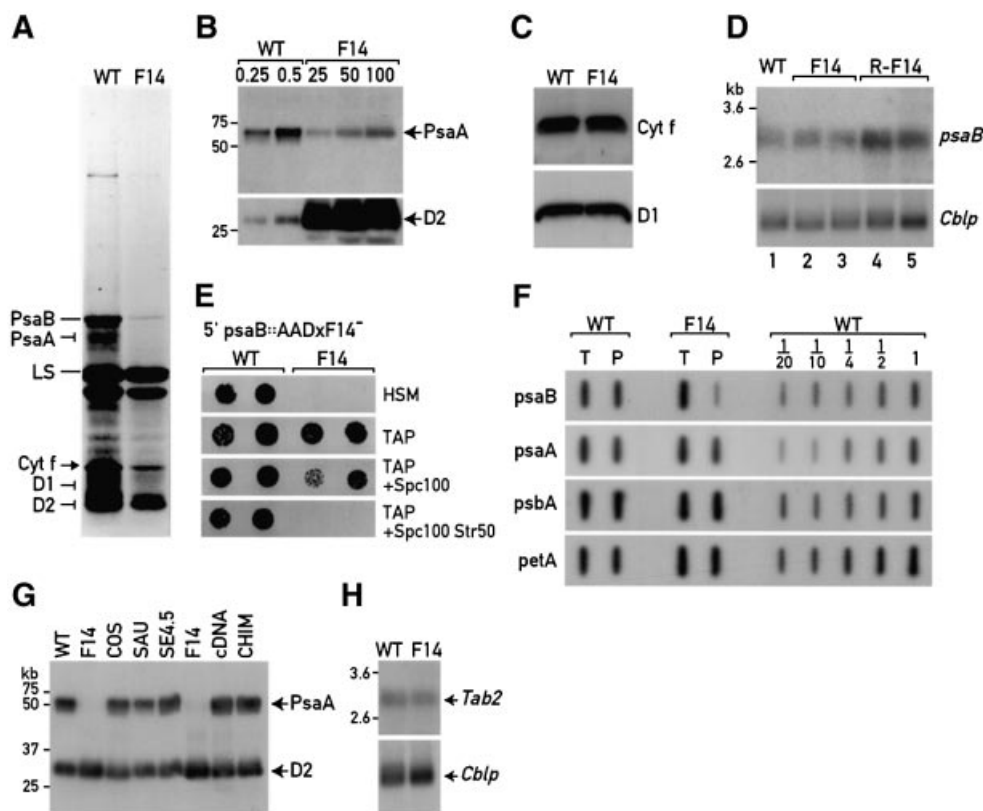
## Introduction

The role of nucleus-encoded factors in the biogenesis of the photosynthetic apparatus has been studied intensively in recent years (reviewed in Barkan and Goldschmidt-Clermont, 2000). Biochemical and genetic approaches in the unicellular green alga *Chlamydomonas reinhardtii* and in the land plants *Zea mays* (maize) and *Arabidopsis thaliana* have revealed the involvement of numerous nuclear genes in post-transcriptional steps of chloroplast gene expression such as RNA processing, RNA stability and translation (for reviews, see Rochaix, 1992, 1996; Mayfield *et al.*, 1995). While most of the nuclear mutations affecting chloroplast post-transcriptional steps in land plants display pleiotropic effects, in *C.reinhardtii* similar mutations have more specific effects on chloroplast

gene expression (Goldschmidt-Clermont, 1998; Leon *et al.*, 1998).

Genetic approaches in *Chlamydomonas* have identified several nucleus-encoded factors involved in chloroplast translation. A characteristic feature of these factors is that they act specifically on single chloroplast mRNAs. Thus, mutants of *Chlamydomonas* have been isolated that are specifically deficient in the translation of the mRNA for *petA* (Wostrikoff *et al.*, 2001), *psaB* (Stampacchia *et al.*, 1997), *psbA* (Girard-Bascou *et al.*, 1992; Yohn *et al.*, 1998) and *psbC* mRNAs (Rochaix *et al.*, 1989; Zerges and Rochaix, 1994; Auchincloss *et al.*, 2002). In most of these cases, the nuclear mutations affect the initiation of translation as shown by reduced expression of chimeric reporter genes driven by the 5'UTRs of these mRNAs in the presence of the nuclear mutant allele. Only one mutant deficient in elongation of *psbD* mRNA translation has been characterized (Cohen *et al.*, 2001). Mutants affected in chloroplast translation have also been identified in maize. Some of these mutants are specifically deficient in the translation of the *atpB/E* mRNA or the *petD* and *petA* mRNAs (Barkan *et al.*, 1994; McCormac and Barkan, 1999; Fisk *et al.*, 1999).

Here we report the characterization of a nuclear gene and its product involved specifically in the initiation of translation of the *psaB* mRNA in *C.reinhardtii*. PsaB and PsaA are the two reaction centre subunits of photosystem I (PSI) that act as ligands of the primary electron donor P700 and the primary acceptors A<sub>0</sub>, A<sub>1</sub> and the 4Fe–4S centre F<sub>X</sub>. The strain carrying the nuclear mutation *F14-tab2* examined here is affected in the synthesis of both PsaA and PsaB. Previous studies showed that mutants deficient in PsaB are unable to synthesize both PsaB and PsaA whereas mutants affected primarily in PsaA synthesis are still able to produce PsaB (Girard-Bascou *et al.*, 1987; Stampacchia *et al.*, 1997). Based on these results it was proposed that PsaB is an anchor protein during PSI assembly, which needs to be synthesized and integrated into the thylakoid membrane before the other PSI subunits are synthesized (Stampacchia *et al.*, 1997). In its absence these polypeptides are no longer synthesized and/or are rapidly degraded. Elucidating how PsaB is translated and inserted into the thylakoid membrane is thus important for understanding the initial steps of PSI assembly. We have characterized *Tab2*, the gene affected in *F14* and we show that it encodes a new type of RNA binding protein that is part of a high molecular weight complex which also includes *psaB* mRNA. The primary effect of the *F14-tab2* mutation is a dramatic decrease in *psaB* mRNA translation. The predicted amino acid sequence of the Tab2 protein displays similarities with several putative proteins found in both prokaryotic and eukaryotic organisms capable of oxygenic photosynthesis and represents to date the first identified factor specifically involved in the



**Fig. 1.** Analysis and rescue of the *F14* mutant strain. (A) Chloroplast protein synthesis in the wild-type (WT) and the *F14* mutant strain. Cells were pulse-labelled with [ $^{14}$ C]acetate for 45 min in the presence of cycloheximide. Thylakoid membrane proteins were separated by gradient 7.5–15% SDS–PAGE. PsaA and PsaB, the two reaction centre polypeptides of PSI; LS, Cyt *f*, D1 and D2 correspond respectively to the large subunit of ribulose-1,5 bisphosphate carboxylase, Cyt *f* and the two reaction centre subunits of PSII. (B) Immunoblot analysis of the wild-type and *F14* strains. Crude cell extracts were separated on a 6% SDS–PAGE and electroblotted. The membrane was reacted with PsaA and D2 polyclonal antisera. The amounts of protein loaded are expressed in  $\mu$ g of total proteins. (C) Immunoblot analysis of 25  $\mu$ g of total proteins from wild-type and *F14* strains with D1 and Cyt *f* antisera. (D) RNA blot analysis of the wild-type, *F14* and rescued *F14* (*R-F14*) strains. Five micrograms of total RNA from these strains were fractionated on a 1% agarose–formaldehyde gel, blotted and hybridized with a  $^{32}$ P-labelled *psaB* probe. Equal loading of mRNA was checked by hybridization of the blot with a *Cblp* probe. Lane 1, WT; lanes 2 and 3, two *F14* isolates; lanes 4 and 5, two independently rescued *F14* transformants (*R-F14*) complemented with the cosmid and pKS-SE 4.5, respectively. (E) Growth patterns of strains containing the chloroplast chimeric *psaB* 5'UTR–*aadA* gene in wild-type and *F14-tab2* mutant nuclear backgrounds. One representative tetrad derived from the cross between *F14* (mt $^-$ ) and the wild-type *psaB* 5'UTR–*aadA* (mt $^+$ ) is displayed (see Materials and methods). WT, progeny with wild-type *Tab2* allele; *F14*, progeny with *F14-tab2* allele; HSM, minimal medium; TAP, acetate-containing medium; Spc100, spectinomycin 100  $\mu$ g/ml; Str50, streptomycin 50  $\mu$ g/ml. (F) 2  $\mu$ g of total RNA (T) and 2  $\mu$ g of polysomal RNA (P) from wild type and *F14* was slot blotted and hybridized with probes specific for *psaB*, *psaA*, *psbA* and *petA*. In each case a wild-type dilution series was used for estimating the amount of RNA. The slot blots used were duplicates of one another. (G) Immunoblot analysis of PsaA polypeptide in wild type, *F14* and rescued *F14*. Total cell protein (5  $\mu$ g) of the wild type (WT), two replicas of the *F14* mutant strain (*F14*) and the *F14* mutant rescued by transformation with the cosmid (COS), the 11 kb *Sau3A* genomic fragment (SAU), the 4.5 kb *SacI*–*EcoRI* minimal genomic fragment (SE4.5), the 2.2 kb *Tab2* cDNA driven by the *psaD* promoter (cDNA) and the chimeric genomic–cDNA *Tab2* gene containing a triple HA epitope (CHIM). D2 antiserum was used to check for equal protein loading. Molecular mass markers are indicated in kDa. (H) RNA gel blot analysis of *Tab2* mRNA in the wild-type and *F14* strains. Five micrograms of total RNA were loaded per lane.

translation and possibly assembly of photosynthetic subunits that is conserved in this broad class of organisms.

## Results

### The *F14* mutant is deficient in the initiation of translation of the chloroplast *psaB* mRNA

The nuclear mutant *F14* was originally described as a high-fluorescence mutant requiring acetate for growth (Bennoun and Levine, 1967) and was classified as a mutant lacking PSI activity. This strain carries a recessive nuclear mutation (Girard *et al.*, 1980). We first tested whether the synthesis of any of the chloroplast-encoded subunits of PSI is impaired in this mutant. Wild-type and mutant cells were pulse-labelled for 45 min with [ $^{14}$ C]acetate in the presence of cycloheximide, an inhibitor

of cytosolic translation, and total cell proteins were analysed by SDS–PAGE and autoradiography. The labelling of the two reaction centre polypeptides of PSI, PsaA and PsaB, is greatly reduced in the mutant (Figure 1A). Moreover, the bands corresponding to cytochrome (Cyt *f*) and the PSII D1 polypeptide are significantly weaker in *F14* than in the wild type.

The level of PsaA protein in the mutant is at least 200 times lower than in the wild type as shown by immunoblotting with serial dilutions of crude extracts (Figure 1B). Because we were unable to raise an antiserum against PsaB, we used a PsaA antiserum throughout this study to measure the amount of PsaB. This is possible because the PsaA and PsaB proteins accumulate to the same level in mutants impaired in the synthesis and/or stability of any of these two PSI reaction centre subunits as

**Table I.** Complementation of the *F14* mutant strain

Plasmid	Size (kb)	Complementation
Cosmid	30	+
pKS-SE4.5	4.5	+
pKS-cDNA	2.2	-
<i>PsaD</i> 5'UTR cDNA	2.2	+
Chimeric gDNA-cDNA	3.6	+
Chimeric gDNA-cDNA::HA	3.7	+/-

Rescue is indicated by '+', lack of rescue by '-', lower rescue efficiency by +/- . pKS-SE4.5 refers to the 4.5 kb *SacI-EcoRI* fragments cloned in pKS-Bluescript; pKS-cDNA, *Tab2* cDNA with partial 5'UTR cloned directly in pKS without *Chlamydomonas* promoter; *PsaD* 5'UTR cDNA, cDNA of *Tab2* cloned in pSL18 driven by the *PsaD* promoter. The chimeric genes result from the fusion of the 4.5 *SacI-EcoRI* genomic DNA containing the *Tab2* 5'UTR and the complete *Tab2* ORF except for the end of the last exon, which has been replaced with the corresponding *Tab2* cDNA sequence.

they form a heterodimer and are stabilized by each other (Girard-Bascou *et al.*, 1987). In contrast to PsaA and PsaB, the apparent reduced synthesis of Cyt *f* and D1 does not prevent the normal accumulation of these polypeptides in *F14* (Figure 1C).

A similar phenotype was observed previously with the non-allelic *F15* mutant (Girard *et al.*, 1980) in which a block in PsaB synthesis leads to impaired PsaA synthesis (Stampacchia *et al.*, 1997). In contrast, mutants deficient in *psaA* gene expression synthesize PsaB protein at the normal rate, although this protein turns over rapidly in the absence of PsaA (Girard-Bascou *et al.*, 1987). From these earlier results we predicted that the primary defect in *F14* is in the expression of *psaB* although the normal accumulation of Cyt *f* and D1 does not rule out the possibility that the factor deficient in *F14* impacts on the translational efficiency of the *petA* and *psbA* mRNAs.

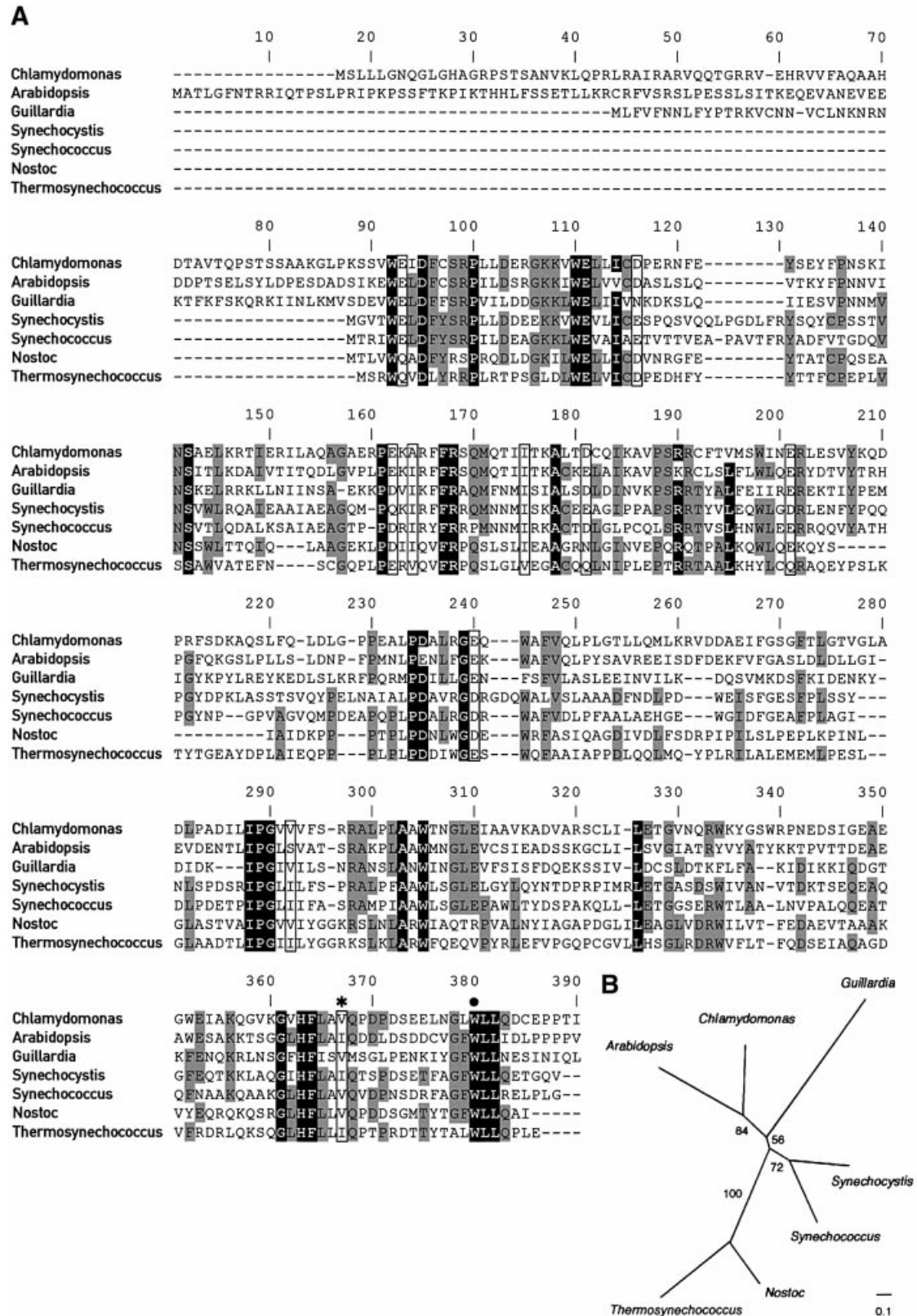
Using RNA blot analysis, we first determined that the *psaB* and *psaA* mRNAs accumulate to normal levels in the *F14* mutant, indicating that the mutation acts at a post-transcriptional step, most likely at the level of translation (Figure 1D, data not shown). To test whether *psaB* is indeed the target of the mutated factor, we used chimeric genes in which the expression of the *aadA* reporter gene conferring spectinomycin and streptomycin resistance (Goldschmidt-Clermont, 1991) is driven by the *psaB* and *psaA* 5'UTRs. These constructs were integrated at the *atpB* locus of the *Fud50* strain as described previously (Nickelsen *et al.*, 1994; Zerges and Rochaix, 1994). The transformants were first selected for growth on minimal medium and then tested for their ability to grow on spectinomycin-containing medium. Once homoplasmy was obtained, the strains were subjected to DNA gel blot analysis and confirmed to contain only copies of the chimeric genes (data not shown). These transformants (mating type +) containing the *psaB* 5'UTR-*aadA* or *psaA* 5'UTR-*aadA* were crossed to the *F14* mutant (mating type -). Because of the uniparental inheritance of the chloroplast genome of *Chlamydomonas* during crosses, all the progeny contain the chimeric chloroplast gene, whereas the nuclear mutation *F14-tab2* segregates 2:2. The progeny of both crosses were scored for phototrophic growth and spectinomycin and/or streptomycin resistance. In the crosses involving the *psaB* 5'UTR-*aadA*-containing

strains, we observed cosegregation between the ability to grow on minimal medium and antibiotic resistance in seven tetrads tested. One illustrative tetrad is shown in Figure 1E. In contrast no effect of the *F14-tab2* mutation was detected on the expression of the chimeric *psaA* 5'UTR-*aadA* gene (data not shown). These results indicate that a target site of the *Tab2* factor is comprised within the *psaB* 5'UTR and that this factor acts most likely at the level of translation initiation. This was tested further by purifying polysomes by sedimentation through a sucrose cushion and by hybridizing total and polysomal RNA with <sup>32</sup>P-labelled probes specific for *psaB*, *psaA*, *psbA* (D1) and *petA* (Cyt *f*) (Figure 1F). As expected the amount of *psaB* RNA associated with polysomes is vastly decreased, <5% of wild-type levels, in the *F14* mutant. In contrast, the level of *psaA* mRNA bound to polysomes in the mutant is reduced only slightly (70% of wild-type levels) and the amount of polysomal *psbA* and *petA* mRNA is the same in the mutant and wild type. To test that the polysomes were intact, their fast sedimentation on sucrose gradients was verified to be sensitive to EDTA which is known to dissociate polysomes (data not shown). Furthermore the bulk of *psbA* mRNA, a representative chloroplast mRNA, was associated with the polysome fraction in the sucrose gradient (data not shown). We thus conclude that the *F14-tab2* mutation impacts principally on translation initiation of *psaB* mRNA. It is likely that other processes are responsible for the decreased signals of PsaA, D1 and Cyt *f* observed during protein pulse-labelling (Figure 1A).

#### ***Tab2* is conserved in oxygenic photosynthetic organisms**

The mutant strain *F14* was rescued by transformation with an ordered cosmid library and selection for phototrophic growth as described (Purton and Rochaix, 1994; Zhang *et al.*, 1994; Vaistij *et al.*, 2000). A cosmid that rescued *F14* was isolated (for details see Supplementary data). Subcloning of this cosmid gave rise to a 4.5 kb *SacI-EcoRI* fragment (pKS-SE 4.5) that was still able to restore the wild-type phenotype by transformation. This fragment was used to screen a *C.reinhardtii* cDNA library. A 2.2 kb cDNA was identified, which, when fused to the *PsaD* promoter and 5'UTR, was able to rescue the mutant. The gene was named *Tab2* for translation of *psaB* mRNA. The efficiency of transformation of *F14* with the different DNA fragments is summarized in Table I. The PsaA protein accumulated to wild-type levels in the various complemented strains as estimated by immunoblotting using an anti-PsaA polyclonal antibody (Figure 1G). As mentioned above, the amount of PsaA protein detected in this blot provides a measure of the amount of PsaB.

Sequencing of the genomic and cDNA fragments revealed that the *Tab2* gene consists of six exons with sizes ranging from 90 to 355 bp and five introns with sizes comprised between 73 and 478 bp. Several stop codons in the same reading frame as *Tab2* are present within the 68 bp sequence upstream of the first ATG detected in the cDNA, indicating that it contains the entire *Tab2* coding sequence. The deduced *Tab2* open reading frame (ORF) encodes a protein of 358 amino acids of 39.9 kDa. A putative chloroplast transit sequence of 50 amino acids rich in arginine and serine residues is predicted by ChloroP. The predicted mature protein consists of 308



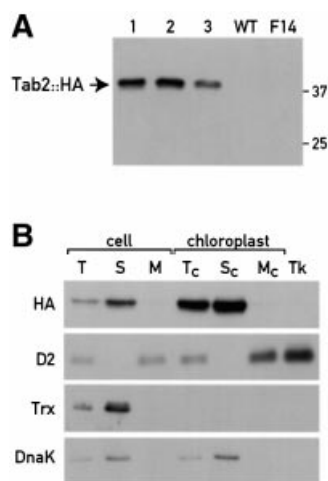
**Fig. 2.** Conservation of Tab2 protein. (A) Sequence alignment of the *C.reinhardtii* Tab2 protein and its orthologues in the land plant *A.thaliana* (At3g08010), the cryptophyte *G.theta* (NP\_113515) and several cyanobacteria including *Synechocystis* sp. (NP\_441046), *Synechococcus* sp. (BAA92865), *N.punctiforme* (ZP\_00112111) and *T.elongatus* (NP\_682776). Sequences were aligned with ClustalW (Thompson *et al.*, 1994). (B) The tree was constructed using protein maximum likelihood with the Molphy program (Adachi and Hasegawa, 1996). Numbers next to branches indicate REML bootstrap proportions (Adachi and Hasegawa, 1996). The scale bar indicates 0.1 substitution per site.

amino acids with a molecular mass of 34.3 kDa and a pI of 5.2. A Blast search did not reveal any similarity with proteins known to be involved in chloroplast translation or with known protein motifs. However, sequence similarity with several proteins of unknown function in both prokaryotic and eukaryotic organisms capable of oxygenic photosynthesis was observed. Figure 2A displays the alignment between the *C.reinhardtii* Tab2 protein and its orthologues in the land plant *A.thaliana*, the cryptophyte *Guillardia theta* and in the cyanobacteria *Synechocystis* sp., *Nostoc punctiforme*, *Synechococcus* sp. and *Thermosynechococcus elongatus*. The phylogenetic tree derived from these alignments is shown in Figure 2B. The eukaryotic sequences are weakly monophyletic with respect to the prokaryotic homologues, suggesting a single origin from the cyanobacterial ancestor of plastids.

Of particular interest is the tripeptide motif WLL near the C-terminal end that is conserved in all Tab2 orthologues. Sequencing of the *Tab2* gene in the original mutant *F14-tab2* revealed a 14 nucleotide insertion 72 bp upstream of the stop codon that resulted in a frameshift. This mutation is expected to give rise to a truncated form of the Tab2 protein in which the 24 C-terminal residues of Tab2 are replaced by a stretch of 14 unrelated amino acids that lacks the conserved tripeptide. RNA blot analysis indicated that the size of the *Tab2* mRNA is 2.8 kb and that the level of this mRNA is similar in the wild-type and *F14* mutant strains (Figure 1H).

### **Tab2 is a chloroplast stromal protein**

Because we were unable to raise an antiserum against Tab2, we constructed a chimeric genomic-cDNA gene containing a triple HA epitope at the 3' end of the coding sequence. This construct was obtained by fusing the 5' part



**Fig. 3.** Localization of the Tab2::HA protein. (A) Immunoblot analysis of total cell proteins (20 µg) of the wild-type, the *F14* and three independent *F14* strains complemented with the HA-tagged chimeric genomic-cDNA *Tab2* gene (lanes 1–3). (B) Subcellular localization of the Tab2::HA protein. Immunoblot analysis of Tab2::HA in whole cells and cellular fractions of *F14* complemented with the Tab2::HA construct. T (total), S (soluble) and M (membrane) extracts; T<sub>c</sub>, S<sub>c</sub> and M<sub>c</sub>, total, soluble and membrane fractions from isolated chloroplasts, respectively. The analysis was also performed on purified thylakoids (Tk). The same fractions were analysed with antibodies directed against the thylakoid D2 subunit of PSII, the cytosolic soluble protein thioredoxin h (Trx) and the chloroplast stromal chaperone DnaK.

of the 4.5 kb genomic fragment described above and the cDNA in frame with the triple HA epitope by using a unique *Bgl*III restriction site in the last exon of the gene. This construct, which is driven by the endogenous promoter and 5'UTR region of the *Tab2* gene, was able to restore phototrophic growth of the mutant after transformation. However, the efficiency of transformation with this HA construct was lower (see Table I). Whole-cell proteins from three photosynthetic transformants were probed by immunoblot analysis with the anti-HA monoclonal antibody (Figure 3A). A 38 kDa protein with the size expected for the HA-tagged Tab2 protein without its transit peptide was detected in all transformants (lanes 1–3) but not in a wild-type strain (lane 4) or in the untransformed *F14* mutant strain (lane 5). Moreover, these transformants containing the HA-tagged Tab2 protein accumulate wild-type levels of PsaA protein (Figure 1G, lane CHIM).

The HA-tagged strains were used to determine the subcellular localization of the Tab2 protein (Figure 3B). The Tab2::HA protein detected in total extract (lane T) was enriched in the soluble fraction (lane S). This protein was undetectable in the membrane fraction (lane M). The Tab2::HA protein was observed in isolated chloroplasts (lane Tc). The lack of cytosolic thioredoxin h (Trx) in all chloroplast-derived fractions (lanes Tc, Sc, Mc and Tk) demonstrates that our chloroplast preparations were not contaminated by cytosolic proteins. Separation of extracts from isolated chloroplasts into soluble and insoluble fractions revealed that the 38 kDa protein is in the soluble fraction (lane Sc). No significant amount of Tab2::HA protein was detected in the chloroplast membrane fraction (lane Mc) or in the purified thylakoids (lane Tk). The soluble chloroplast fraction was not contaminated with thylakoid membranes as indicated by the absence of the PSII D2 protein in lanes S and Sc. The chloroplast stromal DnaK chaperone has a similar distribution to the Tab2::HA protein confirming that the Tab2 protein is present in the same compartment.

### **Tab2 is part of a high molecular weight complex containing *psaB* RNA**

To test whether Tab2 is stably associated with other proteins, the soluble fraction of cells from the *F14* strain complemented with Tab2::HA was subjected to size exclusion chromatography (Figure 4A). The bulk of Tab2::HA protein was detected in fractions corresponding to a size of 300 kDa. However, a significant part of Tab2::HA was also found in the heavier fractions with a size range up to 2000 kDa (Figure 4A). These large complexes were stable in the presence of 2.5 mM EDTA, but destabilized in the presence of 10 mM EDTA (data not shown). In the presence of RNase and MgCl<sub>2</sub>, the higher molecular weight complexes completely disappeared. As a control we verified that the elution of Rubisco was unaffected by the RNase treatment (Figure 4A). These results together with the fact that Tab2 is specifically required for translation of the *psaB* mRNA raised the possibility that the complex contains *psaB* mRNA. To test this, total RNA from each chromatography fraction was extracted, slot blotted and hybridized with a <sup>32</sup>P-labelled *psaB* probe. The bulk of *psaB* mRNA was found in the high molecular weight fractions containing Tab2::HA,

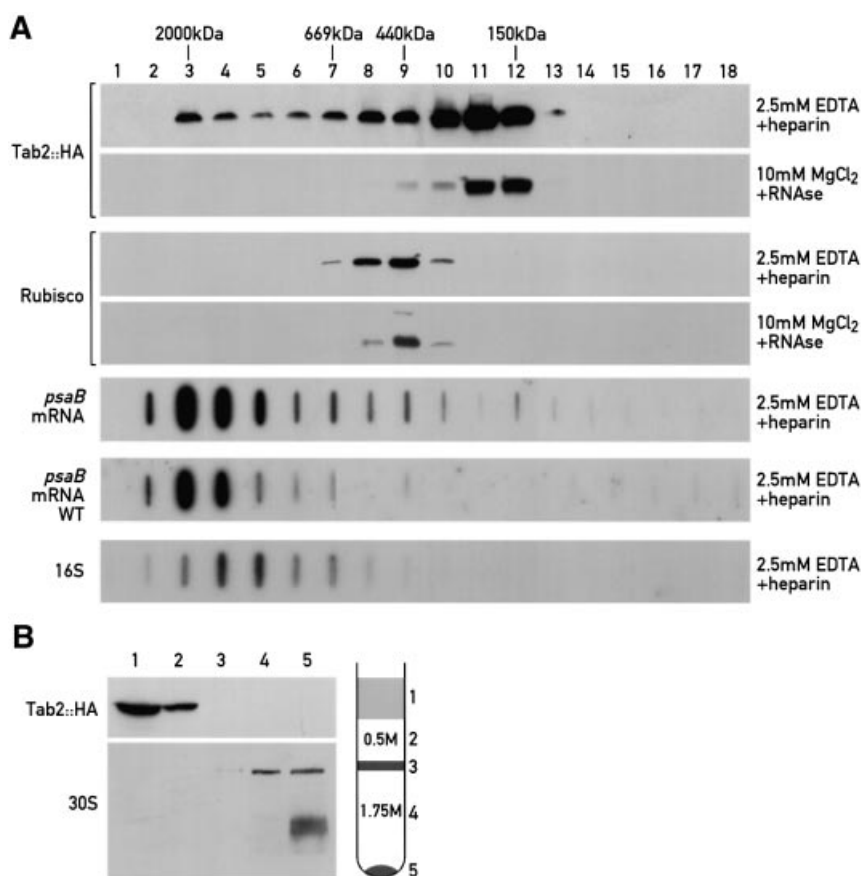
which disappear when the sample is treated with RNase. The same distribution of *psaB* mRNA was found in the wild-type control strain in fractions corresponding to a slightly larger size as compared with the small chloroplast ribosomal subunit containing 16S rRNA (Figure 4A). The Tab2 protein was undetectable in the polysome fraction (Figure 4B, fraction 5).

To confirm the specific association of *psaB* mRNA with the Tab2 complex, a crude extract from the strain expressing the Tab2::HA protein was immunoprecipitated with HA antiserum. RNA was extracted from the immunoprecipitated material and subjected to slot blot hybridization with the labelled *psaB* probe (Figure 5). A signal was readily detected with this probe, but not with a *psbD* probe. Both probes detected their corresponding RNA in the supernatant fraction from the immunoprecipitation (Figure 5). Moreover, when the same experiment was performed with a strain containing the IscU::HA protein, a protein involved in iron–sulfur cluster assembly, no signal was detected with either the *psaB* or the *psbD* probe, indicating that the immunoprecipitation with the HA antiserum was specific (Figure 5). Taken together

these results show that *psaB* RNA is specifically associated with Tab2 in a high molecular weight complex.

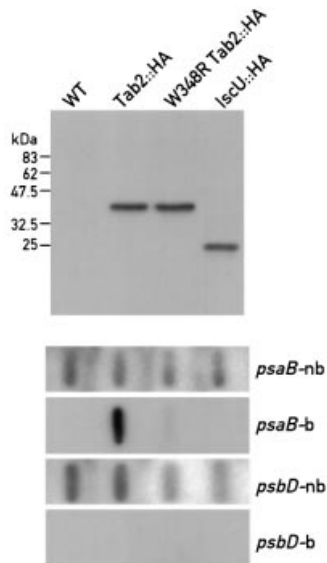
### The W348R mutation abolishes the function of Tab2

The only difference between the wild-type and the mutant *F14-tab2* gene is a 14 nucleotide insertion in the last exon. This insertion creates a premature stop codon and leads to the loss of the C-terminal conserved WLL motif (Figures 2 and 6A). As the mutant strain is not able to grow photoautotrophically, it is possible that this tripeptide motif is essential for the *in vivo* function of Tab2. This was tested by transforming the *F14* strain with mutated versions of the HA-tagged *Tab2* gene. The two constructs tested differ from the wild-type copy by a substitution of W348 by R or by changing the WLL motif to RQQ (Figure 6A). The mutated genomic versions of Tab2::HA were inserted into the plasmid pSL18 and the transformants were selected for resistance to paromomycin (conferred by the *AphVIII* gene present in the plasmid) in low light on acetate-containing medium. Transformants were subjected to immunoblot analysis using the mono-



**Fig. 4.** Tab2 is part of a high molecular weight complex and is not associated with polysomes. (A) Size exclusion chromatography and immunoblot analysis of a cell extract of *F14* complemented with the HA-tagged *Tab2* gene. A soluble fraction was prepared in the presence of either 0.5 mg/ml heparin and 2.5 mM EDTA or 100 µg/ml RNase and 10 mM MgCl<sub>2</sub>, and subjected to size exclusion chromatography. Part of each fraction (20%) was separated by 6% SDS–PAGE, blotted and reacted with HA antiserum. As control the protein blots were performed with Rubisco antiserum. Molecular mass standards are included. The remaining part of the fractions (80%) was used to extract the RNA and analyse the *psaB* mRNA and 16S rRNA distribution by slot blotting. In the case of the *psaB* RNA hybridizations, the slot blots were performed on both the *F14* strain complemented with the HA-tagged *Tab2* gene or a wild-type reference strain. (B) Polysome fractions were immunoblotted with HA antiserum or an antiserum against the chloroplast 30S ribosomal subunit. The drawing corresponds to the classical profile obtained after centrifugation of a soluble crude extract on a discontinuous sucrose gradient (1.75 and 0.5 M). Most of the polysomes are contained in the pellet.

clonal anti-HA antibody and strains expressing the mutated Tab2 protein were examined. Genomic DNA



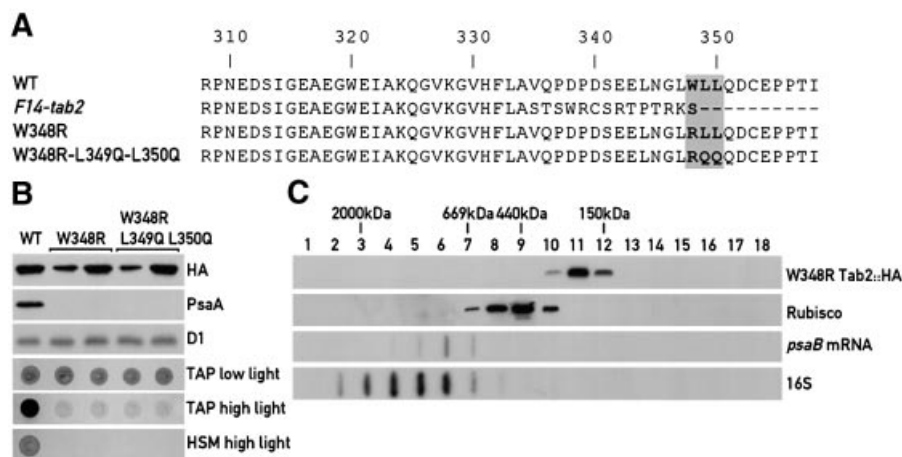
**Fig. 5.** *psaB* RNA is contained within the Tab2 complex. Immunoprecipitation of Tab2::HA and *psaB* mRNA detection by slot blot hybridization. Total soluble extracts were prepared from the *F14* strain complemented with the HA-tagged wild-type *Tab2* gene (Tab2::HA), with the mutant HA-tagged W348R *Tab2* gene, and from a strain carrying an HA-tagged *IscU* gene or from a wild-type strain (control). After incubating the extracts for 1 h at 4°C with HA antiserum bound to beads, the beads were washed and an aliquot (10%) was used to detect the HA-tagged proteins by immunoblotting. The remaining bound material was used for RNA extraction and RNA slot blot analysis with *psaB* and *psbD* probes (*psaB*-b, *psaD*-b). RNA from the non-bound fractions were hybridized with the same probes (*psaB*-nb, *psbD*-nb). The hybridizations with the bound material were overexposed ~40-fold relative to the non-bound material.

from two transformants for each construct was isolated and the presence of the mutation was verified by sequencing. These strains were then tested for photoautotrophic growth on minimal medium (Figure 6B). The selected transformants express the mutated HA-tagged Tab2 protein at levels similar to the wild type. However, both types of transformant containing the two mutant forms of Tab2 were unable to grow photoautotrophically and grew poorly on acetate-containing medium under high light (60  $\mu\text{E}/\text{m}^2/\text{s}$ ). Immunoblot analysis revealed that PsaA does not accumulate to detectable levels in the W348R mutant implying that PsaB expression is affected (Figure 6B). Taken together these results confirm that the deficiency in *psaB* mRNA translation in the mutant is due to the 14 nucleotide insertion near the 3' end of the gene and point to the importance of the highly conserved W348 for Tab2 protein function.

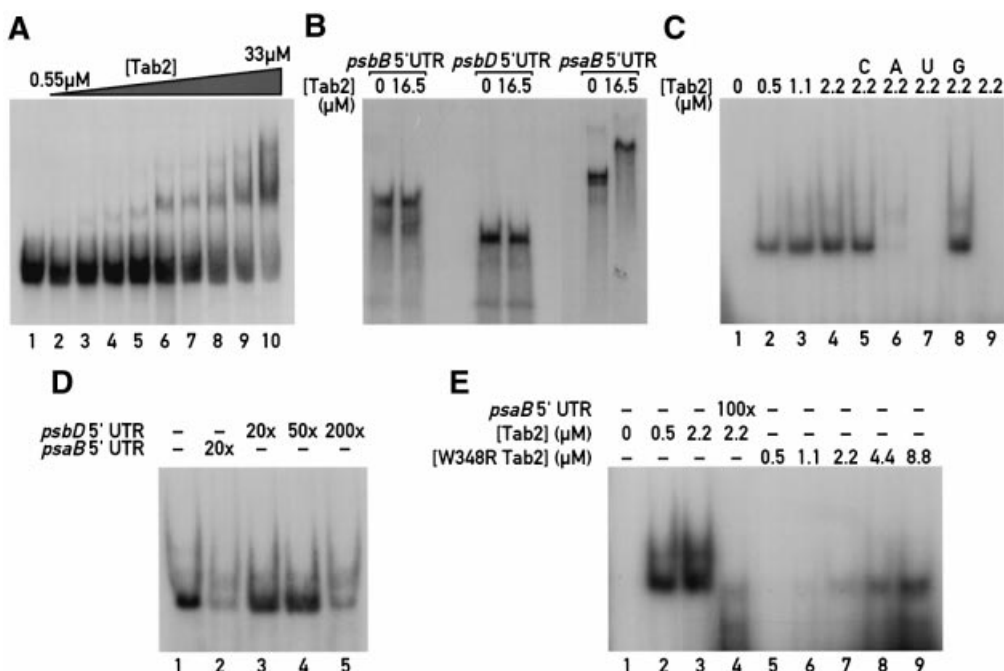
Size exclusion chromatography of a soluble extract from the *F14* mutant strain transformed with a W348R HA-tagged *Tab2* construct revealed that the high molecular weight complex containing the *psaB* mRNA is no longer present and that *psaB* RNA is found in fractions corresponding to smaller sizes (Figure 6C). Moreover *psaB* mRNA was nearly undetectable after immunoprecipitation of extracts from the strain containing the W348R substitution with HA antiserum (Figure 5) further confirming the importance of W348 for the formation of the *psaB* mRNA–Tab2 complex.

#### The Tab2 protein interacts directly with the 5' UTR of *psaB* mRNA in vitro

The dependency of the expression of the chimeric *psaB* 5'UTR–*aadA* gene on the *Tab2* gene and the identification of a high molecular weight Tab2–*psaB* mRNA complex indicate that the Tab2 factor interacts either directly or



**Fig 6.** The WLL motif in Tab2 is essential for its function. (A) Protein sequence comparison between the wild-type Tab2 polypeptide (WT), the putative translated ORF in the *F14-tab2* mutant and the two *Tab2* mutants (W348R and W348R L349Q L350Q). Only the 50 C-terminal amino acids of the proteins are displayed. The complete protein sequence is shown in Figure 2A. (B) Growth patterns of *F14* mutant strains expressing the mutated Tab2 proteins. The *F14* mutant strain was transformed using the HA-tagged chimeric genomic-cDNA described above but containing the amino acid substitutions indicated in (A). The transformants were selected on acetate-containing medium under low light in the presence of paromomycin. Total crude extracts from these strains were used to detect the mutated Tab2::HA protein by immunoblotting. Two independently obtained transformants with each of the two Tab2 mutations were tested for growth on TAP (acetate-containing medium allowing heterotrophic growth) under low and high light and on HSM (minimal medium requiring photoautotrophic growth). The control strain (WT) corresponds to the *F14* mutant strain complemented with the wild-type HA-tagged *Tab2* gene. (C) Gel filtration chromatography of a total soluble extract from the *F14* mutant strain expressing the HA-tagged W348R *Tab2* gene. Immunoblot analysis using the HA and Rubisco antisera and slot blot detection of *psaB* mRNA and 16S rRNA were performed as in Figure 4.



**Fig 7.** Tab2 interacts directly with the *psaB* 5'UTR region. (A) EMSA with recombinant purified Tab2 protein and the 100 3'-terminal nucleotides of the *psaB* 5'UTR. Three femtomoles of *in vitro* transcribed RNA were incubated with increasing concentrations of purified Tab2 polypeptide (0, 0.55, 1.1, 2.2, 3.3, 8.25, 12.4, 16.5, 24.8, 33 μM for lanes 1–10, respectively). (B) The Tab2 protein interacts specifically with *psaB* 5'UTR. EMSA of Tab2 with complete 5'UTRs of *psaB*, *psbB* and *psbD*. Three femtomoles of each probe were incubated without or with 16.5 μM purified Tab2 recombinant polypeptide prior to electrophoresis. (C) EMSA with RNase T1 digestion. After incubation of 3 fmol of *in vitro* transcribed complete *psaB* 5'UTR with Tab2 protein, the samples were treated with T1 RNase prior to electrophoresis (see Materials and methods). Under these conditions, the RNA probe alone is completely degraded (lane 1). In the presence of purified Tab2 protein, a part of the RNA molecule is protected from T1-mediated cleavage. Lanes 2–4 correspond to incubations with 0.55, 1.1 and 2.2 μM Tab2 protein, respectively. Lanes 5–9, competition assays with a 500-fold excess of poly(riboC) (C), poly(riboA) (A), poly(riboU) (U) and poly(riboG) (G) or with 200-fold excess of cold *in vitro* transcribed complete *psaB* 5'UTR (lane 9). (D). T1-EMSA in the presence of cold competitor *psbD* and *psaB* 5'UTR. One femtomole of complete *psaB* 5'UTR was incubated with 2.2 μM purified Tab2 recombinant protein prior to T1 digestion. The competitor RNAs were preincubated with the proteins for 5 min before the labelled transcript was added. The fold excess of each competitor is indicated in the figure. (E) T1-EMSA with wild-type and W348R recombinant Tab2 proteins. Three femtomoles of *in vitro* transcribed complete *psaB* 5'UTR were incubated with different concentrations of purified mutant Tab2 proteins followed by T1 RNase digestion. Lane 1, *psaB* 5'UTR probe without protein; lanes 2 and 3, *psaB* 5'UTR with 0.55 and 2.2 μM wild-type Tab2 recombinant protein, respectively. Lane 4, competition with a 100-fold excess of cold *psaB* 5'UTR. Lanes 5–9, *psaB* 5'UTR with 0.55, 1.1, 2.2, 4.4 and 8.8 μM purified recombinant mutant W348R Tab2 polypeptide.

indirectly with the *psaB* 5'UTR. To distinguish between these two possibilities, electrophoretic mobility shift assays (EMSAs) were performed. The Tab2 protein was expressed in *Escherichia coli* fused with GST and purified using a standard procedure and the GST epitope was removed by thrombin-mediated cleavage. The protein preparation was then incubated with radiolabelled *in vitro* transcribed RNAs corresponding to either the 3' terminal 100 nucleotides of the *psaB* 5'UTR or to the complete *psaB* 5'UTR (495 bases). Formation of an RNA–protein complex with reduced mobility in a non-denaturing polyacrylamide gel was observed with the –100/+1 region of *psaB* 5'UTR with increasing concentrations of purified Tab2 protein (Figure 7A). The first experiments were performed with this sub-fragment of the *psaB* 5'UTR because we expected that the target region of the Tab2 complex would be close to the ribosome binding site. This interaction was confirmed by competition experiments with a 200-fold excess of cold *psaB* RNA (data not shown). Large amounts of recombinant purified Tab2 protein were necessary to completely shift the *psaB* 5'UTR fragment (Figure 7A). We estimate the  $K_d$  of this interaction as ~18 μM based on quantitative measurements of the bands in Figure 7A with a phosphorimager (see

Material and methods). This value appears to be surprisingly high. However, it is possible that the RNA region chosen does not contain the entire RNA binding site. Alternatively it is possible that the recombinant Tab2 protein is not fully refolded or that it needs additional factors for efficient RNA binding. We therefore performed additional mobility shift assays with the complete *in vitro* transcribed radiolabelled *psaB* 5'UTR. In the presence of large amounts of Tab2 (16.5 μM pure recombinant protein per lane) a complete shift could be readily observed with the *psaB* 5'UTR, but not with the *psbB* and *psbD* 5'UTRs (Figure 7B). At this concentration of Tab2 protein (16.5 μM) only half of the –100/+1 region of *psaB* 5'UTR was shifted (Figure 7A, lane 8). The large size of the *psaB* 5'UTR probe prevented clean assays. Further gel shift analysis was therefore performed by treating the samples with T1 RNase prior to electrophoresis (Figure 7C). The interaction between Tab2 and the complete *psaB* 5'UTR was significantly stronger than with the 3'-terminal subfragment (Figure 7A). Competition was tested with a 200-fold excess of cold *psaB* 5'UTR and a 500-fold excess of poly(riboA), poly(riboU), poly(riboC) and poly(riboG). Full competition was achieved with the *psaB* 5'UTR (Figure 7C, lane 9) and with poly(riboU) (lane 7) and to a



lesser extent with poly(riboA) (lane 6). The specificity of this interaction was tested further by competition with the *psbD* 5'UTR. In this case competition was observed only with a 200-fold excess of *psbD* competitor (Figure 7D, lane 5) whereas it occurred with a 20-fold excess of *psaB* competitor (Figure 7D, lane 2).

A purified recombinant Tab2 protein containing the W348R substitution was used in mobility shift assay experiments with the complete *psaB* 5'UTR (Figure 7E). Although the mutated Tab2 protein was still able to interact with the 5'UTR, this interaction was considerably weaker than with wild-type Tab2 (Figure 7E). This result suggests that W348 of Tab2 is involved in the binding of Tab2 to *psaB* mRNA.

## Discussion

### ***Tab2 is an activator of translation initiation of psaB mRNA***

We have characterized the Tab2 factor that is affected in the PSI-deficient mutant *F14*. Protein pulse-labelling of *F14* mutant cells indicates that the synthesis of the two PSI reaction centre subunits PsaA and PsaB is strongly reduced. The finding that the chimeric gene consisting of the *psaB* 5'UTR fused to the *aadA* coding sequence is expressed in the chloroplast in the presence of the wild-type *Tab2* allele, but not in the presence of the *F14-tab2* allele, shows that the *F14-tab2* mutation interferes with *psaB* expression and that at least one target site of Tab2 is comprised within the *psaB* 5'UTR. These results, together with the observation that the fraction of *psaB* mRNA associated with polysomes is strongly diminished in *F14* indicate that the Tab2 factor is involved in the initiation of translation and acts as a translational activator. Interestingly although the protein pulse-labelling results indicate that PsaA synthesis is strongly reduced in *F14* (Figure 1A), a large portion of *psaA* mRNA remains associated with polysomes in this mutant suggesting that translation initiation is only moderately affected and that accelerated protein turnover of PsaA may occur in the absence of PsaB. However, this finding does not agree with results obtained with a chimeric *psaA* 5'UTR-*petA* construct whose expression was shown to be reduced in the absence of PsaB (Choquet *et al.*, 2001). Further studies are required to settle this point.

### ***Tab2 is part of a high molecular weight RNA-protein complex***

Size exclusion chromatography indicates that Tab2 is part of a large complex with a molecular mass close to 2000 kDa. This complex contains *psaB* RNA based on several observations. First, Tab2 and *psaB* RNA cofractionate after size exclusion chromatography. Second, treatment of the complex with RNase A induces a shift in size from 2000 to 350 kDa. Third, *psaB* RNA can be specifically co-immunoprecipitated from cell extracts containing the HA-tagged Tab2 protein with HA antiserum. Fourth, a single amino acid change in Tab2 abrogates the formation of this RNA-protein complex.

The picture that emerges from several recent studies in *Chlamydomonas* and maize is that most of the chloroplast *trans*-acting factors involved in plastid gene expression are part of distinct RNA-protein complexes.

Nac2 and Mbb1, which are involved in *psbD* and *psbB* mRNA processing and stability, respectively, are associated with RNA-protein complexes with sizes ranging from 600 to 1800 kDa. Both of these proteins contain several tetratricopeptide (TPR)-like domains (Boudreau *et al.*, 2000; Vaistij *et al.*, 2000). Raa3, which is required for the *trans*-splicing of the first intron of the *psaA* precursor RNAs, is part of a 1700 kDa RNA-protein complex (Rivier *et al.*, 2001). The Crs1 and Crs2 splicing factors of maize are also part of high molecular weight RNA-protein complexes (Jenkins and Barkan, 2001; Till *et al.*, 2001).

### ***Tab2 is an RNA binding protein conserved in oxygenic photosynthetic organisms***

A rather unique feature of Tab2 is that amongst the ancillary factors involved in plastid gene expression, it is the first to be conserved in both prokaryotic and eukaryotic organisms capable of oxygenic photosynthesis. Our data indicate that Tab2 interacts directly with the *psaB* 5'UTR and thus has to recognize this sequence specifically. However, 5'UTRs are usually poorly conserved between cyanobacteria, algae and vascular plants. If the Tab2 orthologues share the same RNA binding specificity as the *C.reinhardtii* protein, one would have to assume that there is a specific match between each Tab2 orthologue and its RNA binding sequence. It is unlikely that Tab2 is required for translation elongation because it is not associated with polysomes. Another possibility is that the *C.reinhardtii* protein has a dual function by being involved both in the recognition and binding of the *psaB* 5'UTR and in an early step of PSI assembly. Perhaps only the latter role is shared with the Tab2 orthologues from the other organisms. Two specific PSI assembly factors, Ycf3 and Ycf4, have previously been identified in land plants (Ruf *et al.*, 1997), algae (Boudreau *et al.*, 1997) and cyanobacteria (Wilde *et al.*, 1995). However, in contrast to Tab2, these factors act at a post-translational level and are firmly associated with the thylakoid membrane and therefore cannot be part of the stromal Tab2 complex.

### ***How does Tab2 function?***

We propose that Tab2 acts early in the PSI assembly pathway by binding to *psaB* mRNA, perhaps already to the nascent RNA chain. Tab2 could possibly be involved in targeting this RNA to the thylakoid membrane where, together with the thylakoid membrane protein Tab1 (Stampacchia *et al.*, 1997; F.Laroche and J.D.Rochaix, unpublished results), it promotes the initiation of translation and perhaps also the insertion of the nascent PsaB polypeptide chain into the membrane. Shortly before or after initiation of translation, Tab2 must be released from the *psaB* mRNA since it is undetectable in the polysome fraction. At this stage it may recruit a new *psaB* mRNA for translation. Upon insertion of PsaB in the thylakoid membrane, newly synthesized PsaA protein would be stabilized and inserted into the membrane followed by the assembly of the PsaA-PsaB heterodimer that forms the core of the PSI reaction centre.

## Materials and methods

### Strains and media

*Chlamydomonas reinhardtii* wild-type and mutant strains were grown as described (Harris, 1989; see Supplementary data).

### Genomic complementation

The mutant strain *F14* was rescued by transformation with an ordered cosmid library and selection for phototrophic growth as described (Purton and Rochaix, 1994; Zhang *et al.*, 1994; Vaistij *et al.*, 2000; see Supplementary data).

### Nucleic acid techniques

The pSL17 plasmid (S.Lemaire and J.D.Rochaix, unpublished results) containing the *AphVIII* gene conferring paromomycin resistance (Sizova *et al.*, 2001) was used to clone the mutated version of *Tab2::HA*. The pSL18 plasmid (S.Lemaire and J.D.Rochaix, unpublished results) containing both *psaD* 5' and 3'UTRs bordering the multicloning site, and the *AphVIII* gene was used to clone the *Tab2* cDNA. Procedures for standard molecular techniques were performed as described (Ausubel *et al.*, 1998; see Supplementary data). The *Tab2* cDNA and genomic sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AJ577849 and AJ577850, respectively.

### EMSA

For EMSA, between 200 ng and 12 µg of purified *Tab2* recombinant protein (corresponding to concentrations of 0.55–33 µM) were incubated with <sup>32</sup>P-labelled transcript (3 fmol *in vitro* transcribed RNA cloned under control of the T7 promoter in pUC18) in 20 µl of 100 mM KCl, 20% glycerol (v/v), 2 mM EDTA, 20 mM HEPES–NaOH pH 8 and 10 µg of yeast tRNA for 20 min on ice. For competition studies, the competitor RNAs were preincubated with the proteins for 5 min before the labelled transcript was added. In the experiments involving the complete *psaB* 5'UTR, 10 U of T1 RNase (Roche, Mannheim, Germany) were added and the incubation continued for 20 min. Samples were then subjected to electrophoresis (3 h at 200 V and 4°C) in 4% (w/v) native polyacrylamide gel (29:1) using Tris–borate–EDTA as electrophoresis buffer. Gels were dried and exposed at –80°C overnight.  $K_d$  was estimated as the concentration of *Tab2* at which the free and *Tab2*-bound RNA are equal (Figure 7A).

### Protein extracts, size exclusion chromatography and immunoblots

Protein extracts and size exclusion chromatography were performed as described (Zerges and Rochaix, 1998; Rivier *et al.*, 2001; see Supplementary data).

For immunoblot analysis, proteins were separated by SDS–PAGE using an acrylamide concentration of 6% (w/v) and electroblotted to nitrocellulose membranes (Protran, 0.45 µm; Schleicher & Schuell, Inc., Keen, NH). Primary antisera used were mouse α-HA (1:1000, Eurogentec), rabbit α-thioredoxin-h (1:10000; gift of S.Lemaire), α-PsbD (D2) (1:10000; gift of P.Nixon), α-PsaA (1:5000; Redding *et al.*, 1998), α-DnaK (1:1000; H.Naver, K.Wilson and J.D.Rochaix, unpublished results) and α-30S (1:10000, gift of A.Boschetti). The signals were visualized by enhanced chemiluminescence (Durrant, 1990).

Immunoprecipitations of HA-tagged proteins were performed with Anti-HA Affinity Matrix (Roche Diagnostics Corp., IN) following a slightly modified procedure. One hundred micrograms of affinity matrix were first blocked by incubation for 1 h at 4°C with 3 mg of a wild-type total protein extract. The beads were then washed five times with 1 ml of 50 mM Tris–HCl pH 7.5, 150 mM NaCl and 10 mM MgCl<sub>2</sub> and resuspended in 100 µl of the same buffer except that the NaCl concentration was lowered to 100 mM. Subsequently, 3 mg of total protein extract obtained from the HA-tagged transformants were incubated at 4°C for 1 h. The beads were washed five times and resuspended in 200 µl of the same buffer, 20 µl were used for immunoblotting with the monoclonal α-HA antibody and RNA was extracted from the remaining 180 µl and used for slot blot analysis.

### Polysome purification

The polysome fraction was purified as described (Barkan, 1988; Yohn *et al.*, 1996; see Supplementary data).

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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