ATAB2 is a novel factor in the signalling pathway of light-controlled synthesis of photosystem proteins

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Plastid translational control depends to a large extent on the light conditions, and is presumably mediated by nucleus-encoded proteins acting on organelle gene expression. However, the molecular mechanisms of light signalling involved in translation are still poorly understood. We investigated the role of the *Arabidopsis* ortholog of *Tab2*, a nuclear gene specifically required for translation of the PsaB photosystem I subunit in the unicellular alga *Chlamydomonas*. Inactivation of *ATAB2* strongly affects *Arabidopsis* development and thylakoid membrane biogenesis and leads to an albino phenotype. Moreover the rate of synthesis of the photosystem reaction center subunits is decreased and the association of their mRNAs with polysomes is affected. *ATAB2* is a chloroplast A/U-rich RNA-binding protein that presumably functions as an activator of translation with at least two targets, one for each photosystem. During early seedling development, *ATAB2* blue-light induction is lowered in photoreceptor mutants, notably in those lacking cryptochromes. Considering its role in protein synthesis and its photoreceptor-mediated expression, *ATAB2* represents a novel factor in the signalling pathway of light-controlled translation of photosystem proteins during early plant development.

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Introduction

It is now generally accepted that plant chloroplasts are derived from a cyanobacterial ancestor after a single endosymbiotic event, which was followed by an extensive reduction of the plastid genome size. Only about 87 protein-coding genes remain in the *Arabidopsis* chloroplast genome,

most of them related to the photosynthetic electron transfer reactions and to the translation apparatus. In contrast, an estimated ~4500 nuclear genes of cyanobacterial origin encode plastid-targeted proteins (Timmis *et al.*, 2004; Lopez-Juez and Pyke, 2005). An increasing number of these genes have been shown to encode factors acting at various levels of chloroplast gene expression, such as transcription, RNA processing, RNA stability and translation, thus coordinating nuclear and plastid genome expression in response to light or developmental cues (Barkan and Goldschmidt-Clermont, 2000). In particular, translational control plays a major regulatory role in young seedlings. Early biochemical approaches showed that the synthesis of the photosystem I (PSI) and photosystem II (PSII) core proteins is mainly regulated at the translational level in 4-day-old plants and that light induces a general stimulation of chloroplast translation initiation and elongation (Klein and Mullet, 1987; Klein *et al.*, 1988). Some of the nucleus-encoded factors participate in that process as putative translation activators (Bruick and Mayfield, 1999; Choquet and Wollman, 2002). Interestingly, they have been proposed to be involved in the intricate mechanisms that control the translation rate of the subunits of these complexes, in particular those of PSI, of PSII and of the cytochrome *b₆f* complex of *Chlamydomonas reinhardtii* (Choquet *et al.*, 1998; Wostrickoff *et al.*, 2004; Minai *et al.*, 2006). Moreover coordinate accumulation of the subunits of the photosynthetic complexes is achieved at the post-translational level by plastid proteases, which degrade subunits produced in excess. In the case of mutants lacking any of the core subunits, the other subunits are synthesized but rapidly degraded (Barkan and Goldschmidt-Clermont, 2000).

However, although genetic approaches using *Arabidopsis* and maize identified many mutations affecting plastid gene expression, only a few of the corresponding genes have been identified and found to be active in translation in land plants. In *Arabidopsis*, HCF107 is required for *psbB* translation and *psbH* 5'-end processing/stability and, possibly translation. This protein contains 11 TPR-like (tetratricopeptide) repeats that could serve as RNA-binding domains (Sane *et al.*, 2005). In maize, CRP1 is required for *petA* translation, *petD* mRNA processing/translation and presumably also for *psaC* translation (Barkan *et al.*, 1994). Its RNA targets *in vivo* could be identified by immunoprecipitation of protein-bound RNAs with a specific anti-CRP1 antibody, and by labelling and hybridization to a microarray representing the complete chloroplast genome (Schmitz-Linneweber *et al.*, 2005). CRP1 displays 14 tandem PPR (pentatricopeptide) repeats, a type of helical-repeat domain that has been shown to bind chloroplast RNA. Indeed, a direct interaction with chloroplast RNA has been demonstrated *in vitro* solely for the *Arabidopsis* HCF152 PPR protein involved in the processing of the polycistronic *psbB-psbT-psbH-petB-petD* RNA (Meierhoff *et al.*, 2003).

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In order to gain more insights into the translational control of chloroplast mRNAs in plants, we investigated the function of a putative ortholog of the *Tab2* gene, first identified in the unicellular green alga *C. reinhardtii* (Dauvillée *et al*, 2003). In *Chlamydomonas*, the Tab2 protein is specifically required for translation of the *psaB* mRNA that encodes one of the PSI reaction center proteins. Using a genetic approach, the Tab2 target was shown to be within the *psaB* 5'UTR. Moreover, although this protein displays no known RNA-binding domain, it is associated *in vivo* with *psaB* mRNA within a high molecular weight complex and directly binds to the *psaB* 5'UTR *in vitro*. Tab2 is an ancient gene of prokaryotic origin evolutionarily conserved in oxygenic photosynthetic organisms. In particular, it shares significant sequence similarity with a single-copy gene in *Arabidopsis*. This Tab2-like gene (named *Arabidopsis* TAB2 or ATAB2) encodes a protein with 38% and 26% identity with its *Chlamydomonas* and *Synechocystis* PCC6803 orthologs, respectively (Dauvillée *et al*, 2003). *In silico* analysis provides no clues concerning the biochemical role of the encoded protein, except for a potential N-terminal chloroplast transit peptide predicted by TargetP 1.1 (Emanuelsson *et al*, 2000). In the present study we describe the role of ATAB2 as a putative translation activator required for synthesis of both photosystem I and photosystem II in a land plant. During cotyledon expansion, it is rapidly induced through a mechanism involving cryptochromes under blue light. Therefore, considering its function and its photoreceptor-dependent expression, ATAB2 represents a novel factor mediating light-controlled translation of photosystem proteins during early plant development.

Results

Albino phenotype of the *atab2* Mutants

To investigate ATAB2 function in *Arabidopsis*, three T-DNA lines with insertions in its coding sequence (accession At3g08010) were obtained from public collections (Sussman *et al*, 2000; Rosso *et al*, 2003). For simplicity, the GABI-Kat lines 354B01 and 360A10 are respectively referred as *atab2-1* and *atab2-2* alleles while the line WiscDsLox289_292H6 from the University of Wisconsin is referred as *atab2-3* (Figure 1A). The *atab2-1* and *atab2-3* lines each have a single T-DNA insertion, which has been confirmed to map precisely in exons 1 and 3, respectively, by PCR amplification and sequencing of 5' and 3' flanking sequences (data not shown). No significant signal could be detected in protein extracts from *atab2-1* using a rabbit antibody raised against the full-length mature ATAB2 protein, which identifies a band at the expected size of ~35 kDa in WT extracts (Figure 1B).

When grown under normal ($150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) or dim light conditions ($6 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), homozygous plants from all three *atab2* lines display an albino phenotype (Figure 1C). Some of them produced two or four true pale green leaves before stopping further development and no flower and silique could be obtained. As expected for a single recessive mutation, this phenotype segregates in a 1:3 ratio from heterozygous *atab2-1/ATAB2* and *atab2-3/ATAB2* plants, and segregates with a homozygous T-DNA genotype as evaluated by PCR analysis of more than 100 individuals for each mutant. Therefore, albino *atab2-1* plants grown under a fluence rate of $6 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ were used in this study when referred to as *atab2* mutants. Under this

light intensity that avoids indirect effects owing to photo-damage, a five-fold reduction in chlorophyll content and an almost four-fold reduction of carotenoids occur in the mutant (Supplementary Figure S1). Analysis of *atab2-1* mesophyll cells from primary leaves by electron microscopy shows that chloroplasts have an abnormal spherical shape associated with a strong decrease in thylakoid membrane content, and stromal lamellae are nearly absent (Figure 1D). The global size of chloroplasts and the number of stromal plastoglobuli are not significantly changed. A high level of heterogeneity in phenotype could be observed between chloroplasts of different cells, and even between chloroplasts from the same cell. This may be due to a dosage effect of some limiting component required for thylakoid membrane biogenesis.

atab2-1 is affected in PSI and PSII photosystem formation

Light-induced chlorophyll fluorescence measurements indicated that the photosynthetic activity of *atab2-1* leaves is considerably decreased (Supplementary Figure S2A and B). The quantum yield of PSII (Φ_{PSII}) is strongly reduced (0.03 in *atab2-1* versus 0.74 in WT leaves), as well as the ratio of variable to maximum fluorescence (F_v/F_m) that reflects the maximal photochemical yield of PSII (0.51 in *atab2-1* versus 0.77 in WT). The PSII excitation pressure ($1 - q_p$, with q_p indicating photochemical quenching) is considerably higher in the mutant (0.91 versus 0.02 in WT) further indicating that electron flow between PSII and PSI is restricted (Supplementary Figure S2C). Taken together, these data are compatible with a strong defect in PSI or in electron transport between PSII and PSI. Measurements of low-temperature (77 K) fluorescence emission spectra revealed a significant decrease of PSI fluorescence in the mutant (Supplementary Figure S2D) thus indicating a primary defect in PSI and LHCI accumulation (see below).

When tested by immunoblotting, dramatic differences could be observed between *atab2-1* and WT protein extracts (Figure 2A). PSI proteins do not accumulate in the mutant, as shown for the apoprotein PsaA or for the nucleus-encoded PsaD and PsaE subunits. Moreover an ~5-fold decrease of PSII subunits D1, CP47 and OEE1 is also observed. The *atab2-1* mutation specifically affects the photosystems as the levels of subunits of the cytochrome b_6/f complex, the ATP synthase and Rubisco are normal. We have verified that under our laboratory conditions, PSII accumulates in the *Arabidopsis* PSI-deficient mutants *hcf145* (Lezhneva and Meurer, 2004) and *hcf101* (Lezhneva *et al*, 2004) (Supplementary Figure S3). This clearly indicates that the loss of PSI does not necessarily lead to PSII deficiency and that *atab2-1* is atypical. Interestingly, we find that under the same conditions the amount of PSII in *apo1* (Amann *et al*, 2004) is also significantly reduced (Supplementary Figure S3).

The loss of ATAB2 has variable effects on the accumulation of LHCI and LHCII proteins. Overall, LHCII proteins accumulate to 50% of WT levels. This was further tested by probing the blots with antisera specific for individual polypeptides. The levels of Lhcb3, Lhcb5 and Lhcb6 are significantly diminished, whereas Lhcb2 and to a lesser extent Lhcb1 and Lhcb4 are more stable (Figure 2B). In the case of LHCI, Lhca1, Lhca3 and Lhca4 proteins are nearly undetectable, whereas Lhca2 accumulates in limiting amounts (Figure 2B). Altogether, the levels of LCH proteins in the mutant can be

Figure 1 *atab2* T-DNA insertion lines display an albino phenotype and abnormal chloroplasts. (A) Diagram of *ATAB2* gene showing *atab2-1*, *atab2-2* and *atab2-3* T-DNA insertions. Exons are represented by grey boxes pointing in the direction of transcription. Bar = 100 bp. (B) ATAB2 protein is not detected in *atab2-1*. Dilution series of protein extracts (in μ g of protein) from WT and *atab2-1* plants were used for immunoblotting with ATAB2 antiserum. AtpB protein was used as a loading control. (C) Phenotype of 10-day-old mutant seedlings grown under $6 \mu\text{mol m}^{-2} \text{s}^{-1}$ of white light. (D) Chloroplast ultrastructure of first true leaves from WT, *atab2-1* and ATAB2-HA plants analyzed by electron microscopy. Bars indicate the scale.

Figure 2 *atab2-1* is deficient in the accumulation of PSI and PSII. (A) Immunoblot analysis of chloroplast proteins from WT and *atab2-1*. Dilution series of total protein extracts from WT and *atab2-1* 3-week-old seedlings grown under $6 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light were loaded as indicated (μg of protein) and analyzed using the indicated antibodies. (B) Immunoblot analysis of LHCI and LHCII proteins in WT and *atab2-1*. An extract containing $8 \mu\text{g}$ protein was analyzed using peptide antisera recognizing specific LHCI and LHCII proteins. AtpB was used as a loading control.

correlated with the decrease in PSI and PSII and are compatible with the five-fold reduction in chlorophyll.

In agreement with these results, analysis of thylakoid membranes of *atab2-1* by blue native gel electrophoresis revealed that no PSI and PSII complex could be visualized or immunodetected (Supplementary Figure S4A and B). This could either indicate that PSII complex and PSII-LHCII super-complex assembly is impaired in the mutant or, alternatively, that *atab2-1* thylakoid membranes are particularly fragile and lose the PSII complexes during membrane preparation.

Altogether, these data indicate that both photosystems are affected in *atab2-1* seedlings. This shows a clear difference with *Chlamydomonas* in which loss of Tab2 leads to a specific decrease in PSI protein accumulation (Dauvillée et al, 2003).

ATAB2 is a chloroplast protein required during adult plant development

Complementation of the mutant was performed by transforming heterozygous *atab2-1* plants using a *PRO_{35S}:ATAB2-HA* construct in which the *ATAB2* ORF is fused to a C-terminal triple-HA tag. After selfing, several stable lines homozygous for the *atab2-1* mutation and for the *ATAB2-HA* transgene were recovered. One of the complemented lines was used to verify that *ATAB2-HA* is localized in chloroplasts. The protein is present both in the membrane and soluble chloroplast fractions (Supplementary Figure S5). These plants strongly express *ATAB2-HA* in the chloroplast, with a slower PAGE mobility than the mature *ATAB2* protein at $\sim 38 \text{ kDa}$ (Figure 3C). They have a WT phenotype, accumulate normal levels of PsaA and D1 and also develop intact chloroplasts (Figure 1D), indicating that *ATAB2-HA* is able to complement the *atab2-1* mutation.

Among the complemented lines, one exhibited a high level of lethality and no stable double homozygous plant could be recovered (line *ATAB2-HA* #13). Surprisingly, at an

Figure 3 Silencing of *ATAB2-HA* transgene during adult development is lethal. (A) Phenotype of progeny plants obtained by selfing an *atab2-1* + / + *PRO_{35S}:ATAB2-HA* + / - line #13 plant after 7, 21 and 35 days of growth in soil. (B) Chlorophyll fluorescence parameters of WT and of *atab2-1/PRO_{35S}:ATAB2-HA* line #13 plants. ΦPSII , quantum yield of PSII; F_v/F_m , ratio of variable to maximum fluorescence; NPQ, non-photochemical quenching; $1-q_p$, PSII excitation pressure. (C) Immunoblot analysis of total proteins from *atab2-1/PRO_{35S}:ATAB2-HA* line #13 plants. $12 \mu\text{g}$ of protein extracts from *ATAB2-HA* #13 plants with WT (lane 5), yellowish (lanes 6–8) or necrotic (lanes 9–10) phenotypes was loaded. Lanes 1–4, dilution of protein extracts from WT plants. (For colour figure see online version.)

early stage, these siblings homozygous for *atab2-1* had a WT phenotype and strongly expressed the *ATAB2-HA* protein. Because *atab2-1* plants already overexpress the 3' part of *ATAB2* mRNA (Supplementary data), silencing of a second transgene could be facilitated in this genetic background. The progeny of a heterozygous *ATAB2-HA* #13 plant was therefore analyzed further by transferring into soil Basta-resistant seedlings with WT phenotypes, thus harboring at least one copy of the *ATAB2-HA* transgene in a 2:1 ratio. After 3 weeks, one-third of the rosettes were smaller and pale green exhibiting mature cotyledons and adult leaves that displayed necrotic symptoms (Figure 3A). At 5 weeks, they had chlorophyll fluorescence transients similar to the *atab2-1* mutant. This correlates with undetectable levels of *ATAB2-HA* protein, and a strong decrease in PsaA level (Figure 3C, plants 5 and 6). Plants with a milder phenotype (yellowish) had intermediate levels of *ATAB2-HA*, F_v/F_m and ΦPSII values, associated with a high level of non-photochemical quenching (NPQ), suggesting that they are light-stressed under normal light conditions (Figure 3B and C, plants 3 and 4). This delayed

phenotype is apparently owing to the onset of transgene silencing. It suggests that the ATAB2 protein is required not only during the early phase of plant development but also in mature cotyledons and leaves, possibly for the turnover of the photosystems.

***atab2-1* is affected in photosystem protein translation**

Failure to accumulate photosystem proteins could be the direct consequence of a translational defect or due to a deficiency at the level of RNA metabolism. To discriminate between these possibilities, the mRNAs encoding PsaA/PsaB (*psaA-psaB-rps14*), CP47 (*psbB-psbT-psbH-petB-petD*), D2/CP43

(*psbD-psbC*) and D1 (*psbA*) were analyzed by RNA blotting using specific probes (detailed in Supplementary Figure S6A). No significant defect could be observed (Figure 4A). Because most chloroplast mRNA precursors are subjected to complex 5'UTR processing events that can be linked to the formation of translation-competent transcripts (Barkan and Goldschmidt-Clermont, 2000), the 5' ends of the corresponding RNAs were also investigated by primer extension. No major difference could be detected (Supplementary Figure S6B). Therefore, the strong decrease of the PSI and PSII proteins cannot be explained by deficiencies in processing or stability of the corresponding mRNAs.

Figure 4 Accumulation of PSI and PSII proteins is affected at a post-transcriptional level in *atab2-1*. **(A)** RNA gel blot analysis of chloroplast mRNAs in WT and *atab2-1*. Chloroplast mRNAs were detected with the probes indicated on the right and depicted in Supplementary Figure S6A. **(B)** *In vivo* pulse-labelling of membrane chloroplast proteins with [³⁵S]methionine in the presence of cycloheximide. The labelling of PSI (PsaA and PsaB) and PSII (D1, D2 and CP43) proteins is diminished in *atab2-1*. **(C)** *In vivo* pulse-labelling of membrane chloroplast proteins in plants overexpressing ATAB2 (OX). Ten percent of the reactions was also analyzed by immunoblotting using the indicated antisera and AtpB as a loading control (lower panel). **(D)** ATAB2 overaccumulates ~3–4-fold in the OX line as estimated by immunoblot analysis of OX protein extract. AtpB is used as a loading control. **(E)** Association of several chloroplast mRNAs with polysomes is affected in *atab2-1*. Intact polysomes were fractionated by ultracentrifugation and their RNA content analyzed by agarose gel electrophoresis and RNA blotting using the probes indicated. Ribosomal RNAs were stained with ethidium bromide.

In vivo synthesis of chloroplast proteins in *atab2-1* was then monitored by pulse-labelling with [³⁵S]methionine for 5 and 20 min (Figure 4B). As expected, the signals for the α and β subunits of the ATP synthase are unaffected in *atab2-1*. In contrast, the labelling of PsaA and PsaB is drastically reduced and the signals for the PSII subunits D1/D2 and CP43 are also weaker and correlate with their steady-state levels. This suggests that *atab2-1* may specifically affect the translation of some PSI and PSII proteins. Reciprocally, the labelling of the major chloroplast proteins is significantly enhanced in plants overexpressing ATAB2: 1.6-fold for PsaA/PsaB and 2.4-fold for D1/D2 (Figure 4C). The signals for AtpA/AtpB are also significantly increased (1.5-fold), indicating that the effect is not restricted to PSI and PSII proteins. The over-expressing plants accumulate normal levels of photosystem proteins suggesting a post-translational regulation of assembly and/or accumulation of photosynthetic complexes.

Because a reduced labelling of some proteins might also be due to a faster turnover rate, the association of the mRNAs of PsaA/PsaB and D2/CP43 with polysomes was investigated (Barkan *et al*, 1994). Plant extracts were loaded on sucrose gradients under conditions that preserve polysome integrity and mRNAs were fractionated by sedimentation and identified by hybridization with specific probes. The distribution of chloroplast and cytosolic ribosomal RNAs is identical in WT and *atab2-1* extracts (Figure 4E). Also, as expected, the mRNAs of the α subunit of ATP synthase (*atpA*) and cytochrome *f* (*petA*) display a very similar polysome profile in WT and the mutant. In contrast, *psaA/B* and *psbD/C* mRNAs are associated with lighter polysome fractions in *atab2-1* as compared to WT. These data strongly suggest that ATAB2 is a rate-limiting translation activator that acts during initiation and/or early steps of elongation.

ATAB2 is a non-polysomal polyA/U RNA-binding protein

Considering the role that ATAB2 may have in translation initiation, we tested if it is stably associated with ribosomes. A WT leaf extract was fractionated by sucrose gradient centrifugation and collected fractions were analysed by immunoblotting (Figure 5A). Whereas ATAB2 can be detected in the first three fractions, polysomes comprising ribosomal proteins S1 and L1 are found in fraction 5. As previously described (Merendino *et al*, 2003), S1 is also found as a free protein in fractions 1 and 2. Because no signal is observed for ATAB2 in fraction 5, we conclude that ATAB2 does not copurify with polysomes. As expected these polysomes were sensitive to EDTA treatment.

Because the *Chlamydomonas* Tab2 protein binds specifically *in vivo* and *in vitro* to the *psaB* 5'UTR via an unidentified RNA-binding domain (Dauville *et al*, 2003), we examined the RNA-binding activity of ATAB2 *in vitro*. Recombinant GST-ATAB2 protein was expressed and purified from *Escherichia coli*, and the GST epitope was removed by thrombin-mediated cleavage (Supplementary Figure S7A). ATAB2 RNA binding was first tested by RNase T1-EMSA using the *Chlamydomonas* full-length *psaB* 5'UTR for which Tab2 has a higher affinity (Figure 5C). Protection signals are observed with Tab2 and ATAB2 but not with GST protein (grey arrow), whereas the free probe migrating at the top of the gel is entirely degraded. Although higher amounts of ATAB2 are required, a larger complex is observed with both proteins

(white arrows) which may represent either the binding of homomultimeric protein complexes or the binding of several polypeptides at different positions along this 495-nt long RNA substrate. The observation that the two proteins display similar RNA-binding activities for this artificial substrate suggests that ATAB2 function may also involve a direct interaction to its respective RNA targets as previously shown for Tab2.

Because in *Arabidopsis* *psaA* and *psaB* are cotranscribed in a single transcription unit, binding of ATAB2 to the *psaA* 5'UTR was further tested. The formation of RNA-protein complexes was detected with increasing amounts of recombinant ATAB2 but not with GST alone (Figure 5D, white arrow). From these data we estimate the K_d of the RNA binding reactions at approximately 2.2 μ M. Binding to this riboprobe was further tested by T1-EMSA, which gave rise to discrete bands migrating at a similar position as the whole ATAB2-*psaA* 5'UTR complex (Figure 5E). This assay was subsequently used to assess the RNA-binding specificity of ATAB2 by performing competitions with several chloroplast 5'UTRs (*psaA/B*, *psbA*, *psbD/C*, *psbB*, *atpB*), unrelated RNA pBluescript (pBS) and ribo-homopolymers. The results indicate that ATAB2 binds to most of the chloroplast 5'UTRs, but not to the unrelated RNA (Figure 5F). Moreover, ATAB2 binds to poly(rU) and poly(rA) but not to poly(rC) and poly(rG) RNAs (Figure 5G). Examination of the different chloroplast 5'UTRs reveals that they all contain long A/U stretches and that they have an overall low G/C content. From these results, it appears that ATAB2 has a binding preference for A/U-rich sequences at least *in vitro*.

We also tested whether the RNA-binding activity of ATAB2 is influenced by the redox state. No difference was detectable under reducing or oxidizing conditions (Supplementary Figure S7B). In this respect the RNA-binding activity of ATAB2 differs from that of the chloroplast protein RB47/RB60 complex, which was shown to be redox-dependent (Bruick and Mayfield, 1999).

Tab2 function is conserved from *Chlamydomonas* to plants

The possible role of ATAB2 and Tab2 as activators of translation and their 38% amino-acid identity could indicate that Tab2 function has been conserved in land plants. This was tested by a heterologous complementation experiment of *atab2-1*. Remarkably, stable double homozygous plants expressing the *Chlamydomonas* Tab2 protein under the control of a CaMV 35S promoter were recovered. Tab2 is able to complement the *atab2-1* phenotype (Figure 6A and B) to an extent that correlates with its expression level, as estimated with an anti-Tab2 antiserum (Figure 6C, lanes 6 and 7). Indeed, plant development, PSII protein levels and normal chlorophyll fluorescence properties are restored in the line CrTab2#38, although PSI protein PsaA accumulates to ~50% of WT level. A partial complementation was also observed in line #5.

The *Synechocystis* ortholog with 25% sequence identity to ATAB2 was also tested, using a chimeric N-terminal fusion comprising amino acids 1–92 of ATAB2 that includes a chloroplast transit peptide. A C-terminal HA tag was added to monitor its expression, which does not impair ATAB2 function *in vivo*. Although SyTAB2-HA was expressed at a high level, it was unable to complement the PSI and PSII defects of *atab2-1* (Figure 6B and C, lane 8). It appears likely

Figure 5 ATAB2 is not stably associated with polysomes and binds A/U-rich RNAs *in vitro*. (A) ATAB2 does not copurify with polysomal fractions. After centrifugation on a discontinuous sucrose gradient, samples were analyzed by immunoblotting using antibodies recognizing ATAB2 or chloroplast ribosomal proteins S1 and L1. (B) Scheme of *Chlamydomonas* and *Arabidopsis* *psaB* loci with the RNA probes used for *in vitro* binding assays with ATAB2 and Tab2. Riboprobes are represented by thick bars. (C) T1-EMSA using recombinant ATAB2, Tab2 and GST proteins and the complete 495-nucleotide *psaB* 5'UTR of *C. reinhardtii*. The riboprobe was incubated with 12 µg of GST (lane 2) or increasing amounts of ATAB2 (lanes 3–6) and Tab2 (lanes 7–10) polypeptides before RNase T1 cleavage of unprotected RNA fragments. Small RNA-protein complexes are indicated by a grey arrow. c, RNA-protein complexes. (D) EMSA with recombinant ATAB2 protein and the *psaA* 5'UTR of *Arabidopsis*. The riboprobe was incubated with increasing amounts of ATAB2 (0–12 µg for lanes 2–9) or twelve micrograms of GST polypeptides (lane 10) and subjected to EMSA. p, free probe; c, retarded complex. (E) T1-EMSA using recombinant ATAB2 and the *Arabidopsis* *psaA* 5'UTR. When indicated (lanes 4–8), samples were further treated with RNase T1. Under this condition, the riboprobe is almost entirely cleaved leaving a short fragment indicated by a star (*). Amounts of recombinant ATAB2 are in µg. p, free probe; c, RNA-protein complex; pc, protected complex. (F) Competition assay using labelled *Arabidopsis* *psaA* 5'UTR and 10-, 100- or 1000-fold molar excess of cold RNA competitors corresponding to chloroplast 5'UTR mRNAs or a 100-nt pBluescript (pBS) RNA fragment. Band intensities were quantified by phosphorimaging and plotted on the left. (G) Competition assay using labelled *Arabidopsis* *psaA* 5'UTR and increasing amounts of cold poly(ribonucleotides) (rG), (rU), (rC) and (rA). Samples were analyzed as in (F).

that the protein entered the chloroplasts, as two bands with different mobility, corresponding probably to the unprocessed and mature forms, can be detected using the C-terminal HA tag (Figure 6C, lane 8). Partial processing of *Chlamydomonas* chloroplast transit peptides also occurs with the Tab2 protein (lanes 6 and 7).

ATAB2 is induced by blue light during seedling development

Because ATAB2 is required for chloroplast biogenesis, we investigated its expression during early plant development under different light regimes. First, ATAB2 mRNA accumulation was tested in 5-day-old seedlings by RNA blotting

Figure 6 *Chlamydomonas* Tab2 can complement *atab2-1*. (A) Phenotypes of *atab2-1* plants expressing the *Chlamydomonas* Tab2 protein (lines CrTab2#5 and CrTab2#38) or a HA-tagged ortholog from *Synechocystis* PCC 6803 (SyTab2-HA #13). As no stable double homozygous line could be obtained for SyTab2-HA lines, T2 progeny from a heterozygous *atab2-1*/WT plant grown on Basta is shown. WT plants grown on Basta are shown as a control (WT on Basta) to discriminate albino SyTab2-HA plants from Basta-sensitive plants. (B) Chlorophyll fluorescence parameters of complemented plants CrTab2#5, CrTab2#38 and SyTab2-HA. For CrTab2#5 plants, measurements were performed separately on green and variegated plants. SyTab2-HA plants display a typical *atab2* profile. ΦPSII, quantum yield of PSII; F_v/F_m, ratio of variable to maximum fluorescence; 1-q_p, PSII excitation pressure. (C) Immunoblot analysis of total proteins from WT, *atab2-1* and transformed CrTab2#5, CrTab2#38, SyTab2-HA and ATAB2HA plants. Lanes 1–4, dilution of protein extracts from WT plants.

(Figure 7A). It is expressed at a low level in dark-grown seedlings and in seedlings grown under 30 μmol m⁻² s⁻¹ of red light (Figure 7B) or far red light (data not shown). In contrast, its expression is considerably increased in seedlings grown under monochromatic blue light and white light. The level of ATAB2 protein from the same extracts followed changes of its mRNA, with higher levels in light-grown than in etiolated seedlings. In particular, it is higher in seedlings grown under blue than under red light (Figure 7C). Different fluence rates of blue light (1, 10 or 30 μmol m⁻² s⁻¹) were then tested, and a significant induction of ATAB2 expression was observed already with 1 μmol m⁻² s⁻¹ (Figure 7D). It is

noticeable that the amount of ATAB2 is higher in seedlings grown under white light than under blue light, whereas the opposite is true for its RNA level suggesting a more efficient translation under white light. This could also be due to morphologic differences of young 5-day-old seedlings grown under the two different light regimes.

ATAB2 expression was also tested during the de-etiolation response. Four-day-old dark-grown seedlings were exposed for different durations (1, 2, 4 or 18 h) to red, far red or blue light, or maintained in the dark (Figure 7E and F). Under these conditions, ATAB2 mRNA was significantly induced under blue, red or far red light, with the highest level of expression observed after 18 h, the longest exposure tested. Therefore, we conclude that ATAB2 is mainly induced by blue light during early development, whereas during the de-etiolation response all types of light tested are able to trigger its expression. These differences possibly indicate that diverse pathways cooperate to regulate ATAB2 expression during these two developmental phases. In agreement with these experiments we could identify several light responsive elements in the promoter region of ATAB2 (Supplementary Figure S8).

ATAB2 light-dependent expression in photoreceptor mutants

To obtain more insights into the blue-light induction of ATAB2 during early plant development, its expression was examined in mutant plants affected in photoreceptors both at the RNA and protein level. Our analysis was particularly focused on the two *cry1* and *cry2* mutants, as cryptochromes have been shown to be the principal photoreceptors responsible for mediating the blue-light effect on nuclear gene expression (Ma *et al*, 2001; Ohgishi *et al*, 2004). ATAB2 mRNA accumulation was measured by quantitative RT-PCR (qRT-PCR) in plants grown under 30 μmol m⁻² s⁻¹ of blue light as depicted in Figure 7A. After 5 days, the ATAB2 mRNA level increased ~9-fold compared to dark-grown seedlings (Figure 8A). This level was not significantly affected in the phytochrome mutants tested (*phyA*, *phyB* and *phyAphyB*), nor in the double mutant deficient in both phototropin blue-light photoreceptors (*phot1phot2*) nor in the *cry2* mutant. A slight decrease was observed in *cry1*, which was reinforced in *cry1cry2*. Similar patterns were observed at the protein level (Figure 8B). The ATAB2 signal was considerably higher in blue-light-grown than in dark-grown WT seedlings, and weaker signals were observed in *cry1* and *cry1cry2*.

A lower blue light intensity was then tested (2.5 μmol m⁻² s⁻¹), a condition where both CRY1 and CRY2 contribute to photomorphogenesis (Lin *et al*, 1998; Ohgishi *et al*, 2004). Under this fluence, ATAB2 light induction is less pronounced, approximately 4-fold the level of dark-grown seedlings (Figure 8C). Consequently, the difference of expression between different genotypes is also less marked. Nevertheless, a slight reduction of ATAB2 mRNA abundance is observed in the *phyA* mutant and there is a significant decrease in *cry1* and *cry2*. Again, the double mutant *cry1cry2* exhibits the lowest value, reaching only 1.6 ± 0.1-fold the level of etiolated seedlings. Altogether, these data suggest that cryptochromes are important regulators of ATAB2 expression under blue light.

Light-controlled development induces major morphological changes and light-grown photoreceptor mutants have

Figure 7 Seedling development and de-etiolation is accompanied by *ATAB2* induction. (A) Scheme of the experiments in (B–D). Seeds were exposed for 6 h to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of white light to induce germination and exposed to different lights or darkness for 5 days before protein or RNA extraction. (B) RNA gel blot analysis of *ATAB2* mRNA level in 5-day-old seedlings grown under different lights or in the dark. Light intensities are indicated in $\mu\text{mol m}^{-2} \text{s}^{-1}$. (C) Immunoblot analysis of samples in (B). Membranes were incubated with *ATAB2* antiserum, and with DET3 antiserum as a loading control. (D) RNA gel blot analysis of *ATAB2* mRNA levels in 5-day-old plants grown under different intensities of blue light or in the dark. (E) Scheme of the experiment in (F). Seeds were exposed for 6 h to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of white light, grown in the dark for 4 days and then exposed for the indicated durations to $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ of different lights or kept in the dark before RNA extraction. (F) RNA gel blot analysis of *ATAB2* mRNA level during de-etiolation. The durations of de-etiolation are indicated in h. D, Dark; B, blue light; R, red light; FR, far red light; W, white light; ACT, actin loading control.

Figure 8 Expression of *ATAB2* in photoreceptor mutants under blue light. (A) qRT-PCR quantification of *ATAB2* mRNA in 5-day-old seedlings grown under $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light. (B) Immunoblot analysis of samples in (A). Membranes were incubated with *ATAB2* and then with the DET3 antisera. (C) qRT-PCR quantification of *ATAB2* mRNA in 5-day-old seedlings grown under $2.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light. (D) qRT-PCR quantification of *ATAB2* mRNA during the de-etiolation response after exposing dark-grown seedlings to $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light for 18 h. (E) Immunoblot analysis of samples in (D). Membranes were incubated with *ATAB2* and then with the DET3 antisera. qRT-PCR values are normalized relative to *ACTIN2* and the dark-grown control is used as a reference and arbitrarily set to 1. Quantifications of cDNAs were made in triplicate from two biological experiments. Error bars represent $\pm$ s.d.

dramatically different phenotypes under monochromatic light (Neff and Chory, 1998). Experiments bypassing indirect effects caused by the photoreceptor mutations were therefore necessary to determine if *ATAB2* upregulation is due to blue light and cryptochromes *per se*. Because photoreceptor mutants do not have significantly different phenotypes when grown in the dark (Neff and Chory, 1998), *ATAB2* mRNA

expression was investigated during the de-etiolation response (Figure 7E). Four-day-old dark-grown seedlings were exposed to blue light for 18 h, except for the dark control (WT dark). As expected, in dark-grown plants *ATAB2* mRNA level is similar in all genotypes analyzed (data not shown). In contrast, *ATAB2* is upregulated 2.3 ± 0.5 -fold after exposing WT etiolated seedlings to $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light (Figure 8D). Again, its level is diminished in *cry1* and stays at the level of dark-grown seedlings in the *cry1cry2* mutant (Figure 8D). A significant decrease is also observed in *phyA* and *phyAphyB* mutants, which is consistent with previously reported *PHYA*-mediated blue-light perception (Shinomura et al, 1998) and physiological responses (Neff and Chory, 1998). The level of *ATAB2* protein followed changes of its mRNA, with lower levels in *cry* and *phyA* mutants (Figure 8E). These data indicate that *CRY1* and *CRY2* are major players for the control of expression of *ATAB2* by blue light, and that *PHYA* also participates in this process.

Discussion

ATAB2 is involved in the biogenesis of PSI and PSII and acts at the level of translation

We investigated the role of the *Arabidopsis* ortholog of Tab2, a chloroplast protein specifically required for *psaB* translation in the green unicellular alga *C. reinhardtii* (Dauvillée et al, 2003). Development and chloroplast ultrastructure are dramatically affected in light-grown *Arabidopsis atab2* mutants, a feature that highlights the critical role of *ATAB2* during early plant development (Figure 1). At the ultrastructure level, the absence of stromal lamellae is a characteristic of mutants deficient in PSI, as this complex is enriched in stroma-exposed thylakoid regions, whereas PSII is enriched in the appressed regions (the grana stacks) (Andersson and Anderson, 1980). We also observed a strong decrease in the global content of thylakoid membranes. Immunoblot and blue native gel analyses indicate that *ATAB2* is required for the accumulation and/or stability of both PSI and PSII (Figure 2 and Supplementary Figure S4). Inactivation of *ATAB2-HA* transgene expression in adult plants leads to a considerable

decrease in plant size and photosynthetic performance suggesting that ATAB2 is also required in mature tissues, possibly for the turnover or maintenance of PSI and PSII. It seems unlikely that the PSII deficiency in *atab2* is an indirect consequence of the absence of PSI through a feedback retrocontrol. Indeed, some *Arabidopsis* mutants with a drastic deficiency in PSI have normal levels of PSII (Lezhneva and Meurer, 2004; Lezhneva *et al*, 2004; Stöckel and Oelmüller, 2004). However, a significant decrease of PSII is also observed in the PSI mutant *apo1* (Amann *et al*, 2004) although its primary defect is rather different from that of *atab2*.

The loss of most LHCI proteins in *atab2-1* differs from the case of other *Arabidopsis* mutants lacking PSI in which these proteins accumulate at least to some extent, 80–100% in *hcf101* and 25–50% in *hcf145*, but only 10–20% for Lhca2, Lhca3 and Lhca4 in *apo1* (Amann *et al*, 2004; Lezhneva and Meurer, 2004; Lezhneva *et al*, 2004). Although the reason for these differences is not known, these data show that the loss of the PSI core proteins does not necessarily lead to the loss of the antenna proteins. The drastically reduced accumulation of the PSI complex and of the outer PSI and PSII antennae and the decrease of the corresponding fluorescence peaks at 77 K in *atab2-1* raise the possibility that ATAB2 may also be involved in the assembly of the PSI-LHCI supercomplexes and/or in their protection against proteolytic degradation. Finally, we cannot exclude that the loss of the peripheral antenna proteins has some effect on the accumulation of the PSII core.

No significant decrease in the steady-state level and defect in the 5'-end processing of the mRNAs of the PSI and PSII reaction center polypeptides could be detected. Thus, ATAB2 is not required for normal accumulation of these mRNAs. However, synthesis of the PSI apoproteins PsaA and PsaB and, to a smaller extent, of the PSII reaction center proteins D1/D2 and CP43 is diminished in the *atab2* mutant as measured by protein pulse labeling (Figure 4). Moreover, analysis of polysome profiles indicates a defect at the level of initiation of translation or at an early step of elongation in the mutant. These data suggest that ATAB2 most probably acts as a chloroplast translation activator. *In vitro*, the recombinant ATAB2 protein appears to bind preferentially A/U-rich RNA and displays a weak affinity for all 5'UTRs tested. This raises the possibility that its specificity for PSI and PSII is conveyed by some additional factor(s). Alternatively it is possible that the specificity is imparted by the folding of the RNA *in vivo*, which is lost under the *in vitro* conditions used. It is noticeable that overexpression of ATAB2 leads to a general stimulation of synthesis of most major thylakoid proteins. These conditions may favor unspecific binding to other chloroplast 5'UTRs and stimulate the translation of the corresponding mRNAs. Although ATAB2 binding to A/U-rich sequences is compatible with its proposed role in chloroplast mRNA translation, it remains to be seen to which extent this function depends on its RNA-binding activity.

Similar to the CRP1 protein of maize, ATAB2 is not associated to polysomes and may therefore modulate translation efficiency by modifying mRNA secondary/tertiary structures thereby facilitating access of the translation machinery to the 5'UTR (Fisk *et al*, 1999). Alternatively ATAB2 could be loosely associated with polysomes. As proposed for the HCF107 protein, its dual partitioning between stromal and membrane fractions raises the possibility that it associates transiently with the translation machin-

ery (Sane *et al*, 2005) and participates in the early steps of photosystem assembly in the thylakoid membrane.

Evolutionary conservation of ATAB2

Heterologous complementation of an *Arabidopsis* null mutant by the *Chlamydomonas* protein clearly suggests that ATAB2 and *Tab2* are evolutionarily conserved proteins. This successful complementation was rather unexpected, as *Tab2* is only involved in PSI synthesis in *Chlamydomonas* (Dauvillée *et al*, 2003). How *Tab2* succeeds to replace ATAB2 function in photosystem II biogenesis remains to be determined. This could be linked to its overexpression *in planta*, which may favor its binding to RNA targets of lower affinity (Figure 4C). A similar loss of specificity may also occur when ATAB2 is overexpressed. Under these conditions translation of the α and β subunits of ATP synthase is also enhanced besides that of the photosystem core proteins, a process that may be due to increased binding of ATAB2 to A/U rich sequences within these chloroplast 5'UTRs. Alternatively, the RNA targets and/or putative partners of ATAB2 may have evolved differently, making the function of this conserved factor more pleiotropic in land plants. The cyanobacterial ortholog from *Synechocystis* was also expressed in *atab2*, but failed to restore PSI or PSII synthesis, presumably owing to the lower similarity of these two proteins from distant kingdoms.

Unlike CRP1 and HCF107, which belong to the large family of helical-repeat proteins (PPR and RNA-TPR repeats, respectively) that expanded through the plant kingdom (Lurin *et al*, 2004), ATAB2 is an ancient protein of prokaryotic origin with no known RNA-binding domain. ATAB2-mediated regulation of chloroplast biogenesis may thus represent a highly conserved regulatory mechanism that has evolved concomitantly with the chloroplast translation machinery and/or photosystem complexes in photosynthetic eukaryotes.

Photoreceptor-mediated ATAB2 expression

Because the development of plastids in land plants is dependent on light, these organisms offer the possibility to explore the function of nucleus-encoded factors during photomorphogenesis. This developmental program involves rapid thylakoid membrane biogenesis as a consequence of light perception (Lopez-Juez and Pyke, 2005). We observed that ATAB2 is mainly up-regulated by blue light in young seedlings, whereas in de-etiolation experiments, lights of different quality were able to trigger its expression. These differences possibly indicate that diverse pathways cooperate to regulate ATAB2 expression during these two developmental phases. A complex picture of photosensory mechanisms has emerged through which the nucleus- or cytosol-localized photoreceptors integrate light quality, intensity, duration and direction to trigger plant development and adaptation (Gyula *et al*, 2003; Sullivan and Deng, 2003; Chen *et al*, 2004). *Trans*-acting factors like ATAB2 may transduce these signals in the chloroplast.

The response of ATAB2 to low fluence of blue light ($1 \mu\text{mol m}^{-2} \text{s}^{-1}$) and the presence of light-responsive sequence elements in its promoter (Supplementary Figure S8) prompted us to investigate whether blue-light photoreceptors are involved in its regulation. The data obtained support the hypothesis that cryptochromes are important mediators of ATAB2 blue-light induction during early development. However, gene expression profiles indicate that 25% of the

genes that are upregulated when comparing light- and dark-grown seedlings are not induced during transitions in which etiolated seedlings are exposed to light for 36 h (Ma *et al*, 2001). *ATAB2* expression was therefore investigated after de-etiolation for 18 h under blue light, which should also allow one to avoid misinterpretations caused by morphological differences of light-grown photoreceptor mutants. In these conditions, we also observed that *CRY1* and *CRY2* are important for *ATAB2* expression, and that *PHYA* participates in that process. Genome expression analyses indeed support the notion that *CRY1* and *CRY2* regulate similar sets of genes in a partially redundant manner (Wang, 2005). Interestingly, expression of the nucleus-encoded sigma factor AtSig5 and transcriptional activation of its target, the *psbD* Blue Light Responsive Promoter, are also regulated by cryptochromes (Mochizuki *et al*, 2004; Tsunoyama *et al*, 2004). Therefore, *ATAB2* represents a second example of a nucleus-encoded factor of prokaryotic origin transducing a photoreceptor-mediated process in the chloroplast. It could participate in a photoreceptor-mediated signalling pathway activating the translation of photosystem proteins during chloroplast development. It is intriguing that in these two cases, the photoreceptor-mediated blue-light response involves the expression of two proteins, D2 of PSII and PsbA of PSI, which act as anchor proteins in the assembly of their respective complexes. In their absence, translation of the other reaction center partner subunit, D1 for PSII and PsbB for PSI, is repressed through a process elucidated in *Chlamydomonas* and called CES (Wostrikoff *et al*, 2004; Minai *et al*, 2006) and the unassembled subunits are rapidly degraded. Because *ATAB2* is the only plastid translation factor whose expression has been tested for photoreceptor dependence, further studies are required to determine whether this property is unique to proteins that are required for PsbA/PsbB synthesis or whether it is shared by other factors participating in the biogenesis of the photosynthetic apparatus through their involvement in post-transcriptional steps of chloroplast gene expression.

Materials and methods

Plants, growth conditions and transformation

Except *hcf* and *apo1* mutants (accession Wassilewskija, WS), all plants used are in the Columbia (Col) background. Photoreceptor mutants are *phyA-211*, *phyB-9*, *cry1-304*, *cry2-1*, and the double mutants *phyA-211phyB-9*, *cry1-304cry2-1* and *phot1-5phot2-1*. Unless stated otherwise, all experiments were performed using *atab2* and control plants grown on 1.5% sucrose MS medium under 6 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light and 8 h photoperiod. Transformations were performed according to Clough and Bent (1998).

Chlorophyll measurements

Chlorophyll fluorescence inductions were performed with a pulse amplitude-modulated fluorimeter (Hansatech). Photosynthetic

parameters were estimated as described (Meurer *et al*, 1996) and detailed in Supplementary data.

Transmission electron microscopy

Pieces of leaves from 4-week-old WT, *atab2-1* and *ATAB2-HA* seedlings were fixed for 1 h in 2.5% glutaraldehyde, 0.1 M cacodylate pH 7 and 0.001% Tween 20. They were transferred in the same fixative without Tween 20 for 3 h at 4°C. The samples were washed 4 $\times$ 15 min in cacodylate and post-fixed in 1% OsO₄ in cacodylate for 1 h 30 at room temperature. They were rinsed in water and further fixed for 1 h in 1% aqueous uranyl acetate. After washings in water, samples were dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections 60 nm thick were cut and stained with 2% aqueous uranyl acetate and then in Reynolds lead citrate. Sections were viewed in a Phillips EM 10 transmission electron microscope.

Gene expression analysis

For gel blots, RNAs were extracted using the TriReagent (Sigma) and transferred onto nylon N+ membranes (Pharmacia) according to standard procedures. Quantitative RT-PCR analyses were performed using the SYBR green method (Absolute QPCR SYBR Green Fluorescein Mix, Axonlab) and an iCycler iQ Multicolor real-time PCR detection system (BioRad). Detailed information and oligonucleotide sequences are given in Supplementary data.

Protein analysis and antibody production

Plant crude proteins extracts were obtained by grinding tissues in 50 mM Tris pH 8 and 1% SDS and then were centrifugated for 1 min at 10 000 g at 4°C to remove debris and quantified by BCA. *In vivo* pulse-labelling of chloroplast proteins with [³⁵S]methionine, crude polysome extracts and blue native PAGE analysis were performed as described (Barkan, 1988, 1998; Lezhneva *et al*, 2004; Ossendahl *et al*, 2004), respectively, with modifications given in Supplementary data.

For production of *ATAB2* and *Tab2* recombinant proteins, the GST fusion proteins were expressed in *E. coli* BL21, purified and released from the GSH resin by Thrombin-mediated cleavage as described (Dauvillée *et al*, 2003). To obtain the *ATAB2* and *Tab2* antisera, the recombinant proteins were further purified on a 15% SDS-PAGE, resuspended in 15 mM ammonium carbonate, 0.025% SDS, 1 mM DTT and 0.1 mM PMSF after electro-elution and injected in a rabbit five times at 3-week intervals.

RNA-binding assays by EMSA and T1-EMSA were performed as described (Dauvillée *et al*, 2003). *In vivo* pulse labelling experiments were performed as described by Amann *et al* (2004) and polysome analysis as described by Barkan (1988, 1998). Detailed information is given in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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