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Ribosome and transcript copy numbers, polysome occupancy and enzyme dynamics in *Arabidopsis*

Maria Piques, Waltraud X Schulze, Melanie Höhne, Björn Usadel, Yves Gibon[1], Johann Rohwer[2] and Mark Stitt*

Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, Potsdam-Golm, Germany
[1] Present address: INRA Bordeaux, University of Bordeaux 1&2, UMR619 Fruit Biology, F-33883 Villenave d'Ornon, France
[2] Permanent address: Triple-J Group for Molecular Cell Physiology, Department of Biochemistry, Stellenbosch University, Private Bag X1, 7602 Matieland, South Africa
* Corresponding author. Max Planck Institute of Molecular Plant Physiology, Am Muehlenberg 1, Potsdam-Golm 14474, Germany. Tel.: +49 331 5678100; Fax: +49 331 5678101; E-mail: mstitt@mpimp-golm.mpg.de

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Plants are exposed to continual changes in the environment. The daily alternation between light and darkness results in massive recurring changes in the carbon budget, and leads to widespread changes in transcript levels. These diurnal changes are superimposed on slower changes in the environment. Quantitative molecular information about the numbers of ribosomes, of transcripts for 35 enzymes in central metabolism and their loading into polysomes is used to estimate translation rates in *Arabidopsis* rosettes, and explore the consequences for important sub-processes in plant growth. Translation rates for individual enzyme are compared with their abundance in the rosette to predict which enzymes are subject to rapid turnover every day, and which are synthesized at rates that would allow only slow adjustments to sustained changes of the environment, or resemble those needed to support the observed rate of growth. Global translation rates are used to estimate the energy costs of protein synthesis and relate them to the plant carbon budget, in particular the rates of starch degradation and respiration at night.
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Introduction

Plant growth is driven by photosynthetic assimilation of carbon (C). Nutrients like nitrate are absorbed by the roots and converted to amino acids in the leaves using light energy, or imported C in the roots. Sucrose and amino acids are transported to the shoot and root apex to support growth of more leaves and roots. Plants are unavoidably exposed to changes in the environment. One of the most striking changes is the daily alternation of light and darkness, which leads to an extreme and repeated alternation between two states, occurs every day in the natural environment, and can be precisely simulated in laboratory experiments. It results in a large positive balance of energy and C in the light period, and a deficit in the dark period. This is buffered by storing some of the newly fixed C as starch, and remobilizing it to support metabolism and growth during night (Geiger *et al*, 2000; Smith and Stitt, 2007). However, we do not know how energetically expensive processes like protein synthesis are regulated during these marked diurnal changes in the plant's energy budget.

Plants are also exposed to slower changes that occur in a time range of days or weeks, as a result of changing weather patterns and seasonal changes. A very large portion of the total leaf protein is invested in a single metabolic process. Owing to its very low rate of catalysis (K_{cat}=3 s^{-1}), RubisCO represents over 30% of the total protein in a leaf (Farquhar *et al*, 2001; Zhu *et al*, 2007). Large amounts of protein are also invested in the synthesis of chlorophyll-binding proteins and other enzymes involved in the Calvin cycle and pathways for carbohydrate and amino acid synthesis. This raises the question about how plants integrate their response to the environment over a wide range of time spans to generate appropriate levels of proteins for photosynthesis and growth.

Thousands of genes undergo diurnal changes of their transcript level (Bläsing *et al*, 2005) driven by the circadian clock, light and sugars (Usadel *et al*, 2008). This includes many transcripts that encode enzymes in central metabolism. Gibon *et al* (2004b) developed a robotized platform to profile the maximum activities of over 20 enzymes, most of which show rather small diurnal changes (Smith *et al*, 2004; Gibon *et al*, 2004b). Although transcripts respond within hours, changes of

enzyme abundances require days to adjust when plants are transferred to continuous darkness (Gibon *et al*, 2004b, 2006). Gibon *et al* (2004b) hypothesized that the rate of translation is so slow that several days are required to produce a major change in protein abundance. As a result, the rapid transient changes of transcripts would be integrated over a longer period of time to set the levels of enzymes and other proteins. This would buffer the enzymatic capacities in central metabolism against recurring changes caused by the light–dark cycle, while allowing them to adjust to sustained changes in the surroundings.

Protein synthesis occurs by the recruitment of transcripts to ribosomes, to form polysomes. Its synthesis represents a major portion of the total ATP consumption in animal and plant cells (Hachiya *et al*, 2007; Pace and Manahan, 2007; Proud, 2007). Energy is also required for the synthesis of amino acids. The conversion of nitrate to amino acids requires about five ATP molecules per amino acid (Penning de Vries, 1975; Hachiya *et al*, 2007). The synthesis of ribosomes requires energy, and diverts resources from other cellular components. Eukaryotic ribosomes typically contain one molecule of each of the four different ribosomal RNA (rRNA) species and one molecule of ca. 80 different ribosomal proteins (Perry, 2007). Ribosomal RNA and proteins represent >80 and 30–50% of the total RNA and protein, respectively, in a growing yeast cell (Warner, 1999; Perry, 2007). There is selective pressure to achieve a parsimonious use of the translational machinery (Beilharz and Preiss, 2007; Lackner *et al*, 2007). In budding yeast, up to 85% of the ribosomes are present in the polysomes. The ribosome density in polysomes is about one-fifth of the theoretical maximum (Arava *et al*, 2003; MacKay *et al*, 2004), which is consistent with the view that translation is generally regulated by the rate of initiation. A twofold decrease of transcript levels for some ribosomal proteins leads to a ribosome deficit and a *minute* growth reduction phenotype in *Drosophila*, indicating that the overall ribosomal number limits protein synthesis and growth (Perry, 2007).

The rate of translation of a given transcript species depends on transcript abundance, the proportion present in polysomes (often termed 'ribosome occupancy'), the number of ribosomes present on the transcript (often termed 'ribosome density'), and their speed of progression along the transcript (Arava *et al*, 2003; Beilharz and Preiss, 2004; Beyer *et al*, 2004; Brockmann *et al*, 2007). On average, about 70% of a given transcript species is occupied by ribosomes. Similar percentages of ribosomes (60%) and transcripts (59–82%) are loaded into polysomes in plants (Kawaguchi *et al*, 2004; Kawaguchi and Bailey-Serres, 2005).

In the following article, we describe the methods that allow quantitative analysis of ribosome and transcript concentrations, and polysome composition in *Arabidopsis* rosettes. These data allow us to predict translation rates, both globally, and for individual enzymes in central metabolism. We explore the consequences of these molecular events for important subprocesses in plant growth. First, we compare the rates of synthesis with the protein abundance to predict which enzymes are likely to be subject to rapid turnover. Second, we use these molecular data to estimate the costs of protein synthesis and relate to the C budget, in particular the rates of starch degradation and respiration at night.

Results

Experimental strategy

Figure 1 outlines our experimental strategy. Ribosome copy numbers are measured using quantitative real time RT–PCR (qRT–PCR) for rRNA, and polysome fractionation was used to estimate the proportion actively involved in translation. This information is used to estimate the overall rate of protein synthesis. qRT–PCR is combined with polysome fractionation to estimate the copy number of transcripts in polysomes, including 84 that encode enzymes involved in central plant metabolism. This information is used to estimate the rates of translation of individual transcripts. In parallel, quantitative proteomics and robotized measurements of maximum enzyme activities are used to provide two independent estimates of the amounts of these enzymes in the leaves. By comparing the estimated rates of synthesis with the measured abundance of total and individual proteins, and the rate of growth, it is possible to predict whether the global rate of protein synthesis and the rates of synthesis of individual proteins are of the same order as that requited for growth, or whether proteins are subject to rapid turnover.

Polysome fractionation

In preliminary experiments, we collected whole rosettes from 5-week-old wild-type *Arabidopsis* growing in a 12-h light–dark cycle at 6 times during the diurnal cycle, fractionated the material by centrifugation, and collected three fractions: the

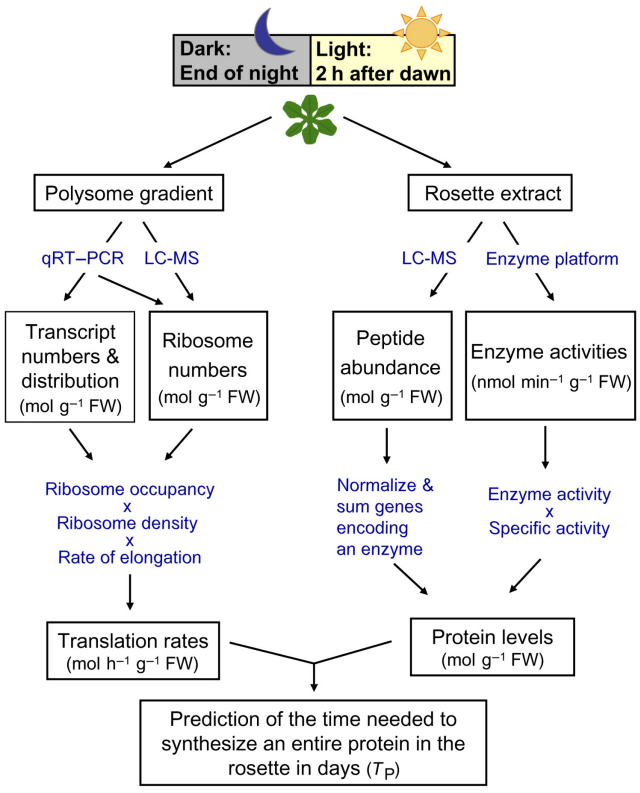

Figure 1 Quantitative analysis of translation in *Arabidopsis* rosette leaves.

Table I Ribosome content in the cytosol, plastids and mitochondria

	Ribosome content (mol g^{-1} FW)	
	Dark period	Light period
Cytosol	7.62E−11 ± 1.56E−11	7.27E−11 ± 1.79E−11
Plastid	2.64E−11 ± 6.66E−12	2.57E−11 ± 7.42E−12
Mitochondrion	2.25E−12 ± 4.42E−13	2.21E−12 ± 3.96E−13

The results are represented as mean ± s.d. of three biological samples. Estimation was done by qRT–PCR of *SSU* rRNA subunits of the cytosolic, plastidic and mitochondrial ribosomes.

non-polysomal fraction (NPS) at the top of the gradient, the small polysomal fraction (SPS) with an estimated 2–4 ribosomes per polysome, and the large polysomal fraction (LPS) with an estimated ⩾5 ribosomes per polysome. The distribution of total RNA in polysome gradients was monitored by measuring A_{254} (Supplementary Figure 1). The proportion in the LPS decreased by about twofold during the night, and recovered within 2 h in the next light period (Supplementary Figure 2). In subsequent experiments, plants were collected at the end of the night ('dark') period and after 2 h of illumination ('light'), corresponding to the largest changes in polysome abundance.

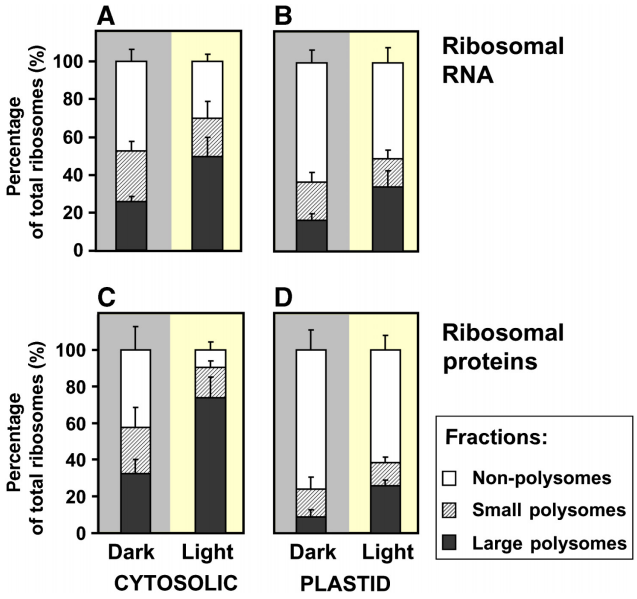

Figure 2 Distribution of ribosomes in different polysomal fractions in the dark and the light periods. (**A**, **B**) Ribosome number in each fraction was calculated by determining the amount of the *SSU* rRNA for cytosolic and plastid ribosomes by qRT–PCR, assuming each rRNA copy corresponds to one ribosome. (**C**, **D**) Ribosomal protein abundance in each fraction, calculated by normalizing the summed emPAI values for ribosomal proteins on total protein in the fraction.

Quantification of ribosomes

A_{254} does not distinguish between transcript RNA and rRNA, and does not distinguish between cytosolic, plastid and mitochondrial rRNA. Two complementary approaches were taken to quantify cytosolic and plastid ribosomes.

The first approach used qRT–PCR to determine the concentrations of 18S, 16S and 18S-like rRNAs, corresponding to the rRNA in the small subunit of the cytosolic, plastid and mitochondrial ribosomes, respectively. To allow precise quantification, the qRT–PCR data were normalized on four artificial control RNAs, which were added at a known concentration before purification of the rRNA from the gradient fractions. The total estimated concentration of ribosomes is about 0.10 nmol g^{-1} fresh weight (FW) (see, Table I and Calculations and assumptions section). Cytosolic ribosomes were threefold more abundant than plastid ribosomes, and 30-fold more abundant than mitochondrial ribosomes. This is in agreement with earlier reports that 26–36% of the total ribosomes in leaves are present in plastids (Dyer *et al*, 1971). The distribution of different rRNA species in the polysome gradient suggests that about twofold more ribosomes were present in the polysomes in the light period than in the dark period (Figure 2A and C). The proportion of cytosolic rRNA in the LPS fraction increased from 26 to 50%, whereas the proportion in SPS decreased slightly (from 27 to 20%), as expected in the case if the polysome population is shifting towards a higher number of ribosomes per transcript, and free cytosolic ribosomes decreased from 47 to 30%. A qualitatively similar picture was found for plastid rRNA, except that a smaller proportion was found in polysomes (Figure 2B). This conclusion is supported by the distribution of ribosomal proteins (Figure 2D). The distribution of mitochondrial rRNA was not investigated.

The second approach used relative quantitative proteomics based on emPAI values (Ishihama *et al*, 2005) to estimate the abundance of ribosomal proteins in the polysome gradient fractions (Figure 2C and D). Briefly, the number of identified peptides per protein are corrected for the number of possible peptides from that protein, and taken as quantitative measure of the protein abundance (see Calculations and assumptions section). This approach confirmed that about twofold more ribosomes are present in polysomes in the light period than in the dark period, and that a larger proportion of cytosolic ribosomes than plastid ribosomes are present in polysomes. The measurements of protein abundance indicate a higher proportion of ribosomes in polysomes than the measurements of rRNA for cytosolic ribosomes. This might be for technical reasons. The NPS fraction is a complex matrix with a high proportion of proteins with other biological functions and emPAI may underestimate ribosomal proteins, especially in the light period, when they are strongly depleted in NPS.

Taken together, both approaches reveal marked changes in ribosomal loading into polysomes between the dark period and the light period, with 53–58% of the cytosolic ribosomes being loaded in the dark period, and 70–90% in the light period. The values in the light are similar to those in rapidly growing yeast (see Introduction).

Global rates of protein synthesis compared with growth requirements

Information about the numbers of ribosomes and the proportion found in polysomes was used to estimate the overall rate of protein synthesis. The summed concentration of

cytosolic, plastid and mitochondrial ribosomes is about 0.1 nmol g^{-1} FW (see Table I). Assuming that a ribosome adds 3 amino acids per s (see Calculations and assumptions section), these could catalyze the addition of 26 µmol amino acids per g FW day^{-1}, equivalent to the synthesis of about 3 mg protein g^{-1} FW day^{-1}. The actual rate of protein synthesis is lower, because only 70% of the ribosomes are in polysomes in the light period, and 40% in the dark period (Figure 2), resulting in an estimated synthesis rate of 1.8 mg protein g^{-1} FW day^{-1}. Under the conditions used in these experiments, *Arabidopsis* contains about 15 mg protein g^{-1} FW (Gibon *et al*, 2004a, 2009; Hannemann *et al*, 2009) and grows exponentially (see e.g. Tschoep *et al*, 2009), with a relative growth rate of 0.15–0.20 g FW g^{-1} FW day^{-1}, which is equivalent to the synthesis of 2.2–3.0 mg protein g^{-1} FW day^{-1}. The rate of protein synthesis estimated from ribosome copy number therefore resembles that required for the observed rate of growth.

Ribosomal occupancy of transcripts encoding enzymes in central metabolism

We next investigated ribosomal occupancy of transcripts for 98 genes (Supplementary Table I) including the major members of the gene families for 35 enzymes of central metabolism (84 transcripts). We also included *AtCAB1* and *AtCAB2* as representatives of photosynthetic and circadian-regulated genes whose expressions peak at the beginning of the day (Ernst *et al*, 1990), three circadian-regulated genes whose expressions peak at the end of the day (*GER3*, *CAT3*, *GRP7)* and 9 transcripts for 'house-keeping' genes that are frequently used for normalization of transcriptional analyses (Czechowski *et al*, 2005). They are all nuclear encoded, except for the plastid-encoded RubisCO large subunit (*RBCL*).

We used qRT–PCR to investigate the distribution of these 98 transcripts in the polysome gradients. To allow precise quantification, the qRT–PCR data were normalized on four artificial control RNAs that were added at a known concentration before purification of RNA in the gradient fractions. This allowed copy numbers of each transcript species to be determined per fraction. In addition to being necessary for subsequent calculations (see below), this bypassed the problems associated with normalization of transcript levels between fractions in polysome gradients. Transcript levels are usually normalized to total RNA. However, the relative levels of transcript and rRNA probably change across a polysome gradient. 'House-keeping' genes are often used for normalization of qRT–PCR data between different organs or environment treatments, but cannot be used for this purpose in polysome fractions, because it is not known whether they are subject to translational regulation. The proportion of transcript in each fraction of the polysome gradient can be easily calculated using qRT–PCR data in combination with internal standards, by simply comparing the numbers of transcripts against internal standard as a control across the whole gradient. The transcript concentrations in the rosette (unfractionated RNA) and polysome fractions, and the ribosomal occupancy (i.e. the proportion of a transcript in the SPS and LPS fractions) are provided in Supplementary Table II, along with the information about the length of the coding sequence, and length and molecular weight of the encoded peptide.

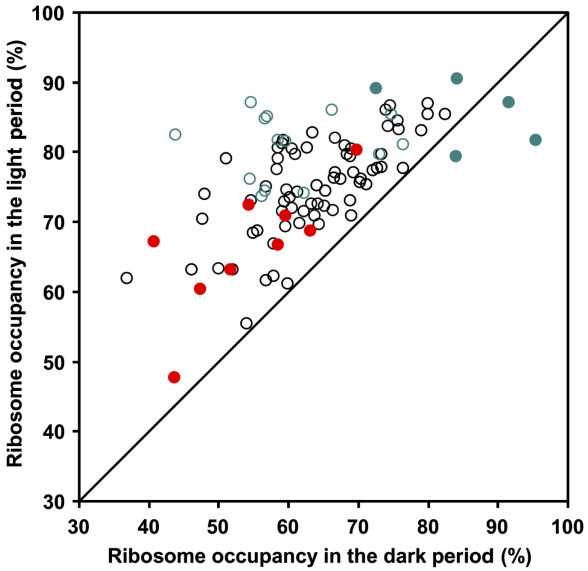

Figure 3 Scatter plot comparing the ribosome occupancy of 98 transcripts in the night and in the light period. Ribosome occupancy was calculated as (SPS + LPS)/(NPS + SPS + LPS). Green circles represent photosynthetic proteins, green filled circles indicate *RBCS* gene family and *RBCL*, and red circles indicate genes that are classified as 'house-keeping' in expression studies. The plot is generated using data provided in Supplementary Table II.

Ribosomal occupancy of the 98 transcripts analyzed, varied between 40–95% in the dark period, and 50–90% in the light period (Figure 3). Most transcripts show a marked increase in occupancy between the dark and the light periods, with an increase of between 5 and 55% in absolute terms, and between 10 and 100% relative to the value in the dark period. 'House-keeping' genes showed a similar response to other transcripts. *RBCL*, *RBCS-1B* and *RBCS-2B* did not show any increase in ribosomal occupancy in the light (see below for further discussion). The increase in ribosomal occupancy in the light is smaller than the twofold increase of ribosome in polysomes (Figure 2), indicating that the average number of ribosomes per transcript probably increases in the light period.

Ribosomal occupancy was weakly but significantly dependent on transcript concentration, with a Pearson's R^2 value of 0.065 (P=0.011) in the dark period, and 0.102 (P=0.001) in the light period (Figure 4). Transcript concentrations varied by three orders of magnitude. There was a large range of ribosomal occupancy (from 40 to >80%) for transcripts with the same concentration. Some of this was due to the effect of light, but there was still a large range when transcripts are considered in one condition. This indicates that ribosomal occupancy depends more on individual features of transcripts than their concentrations.

For almost all transcripts except those encoding RubisCO subunits, there are 10–100 times lesser transcripts in the SPS fraction than in the LPS fraction. This is shown by the low $\log_{10}$ ratio of SPS/LPS in Figure 5. The proportion of transcript in SPS compared with LPS decreases as the proportion in the NPS fraction decreases, resulting in a near-linear relation in this semi-log plot. This empirical relationship indicates that

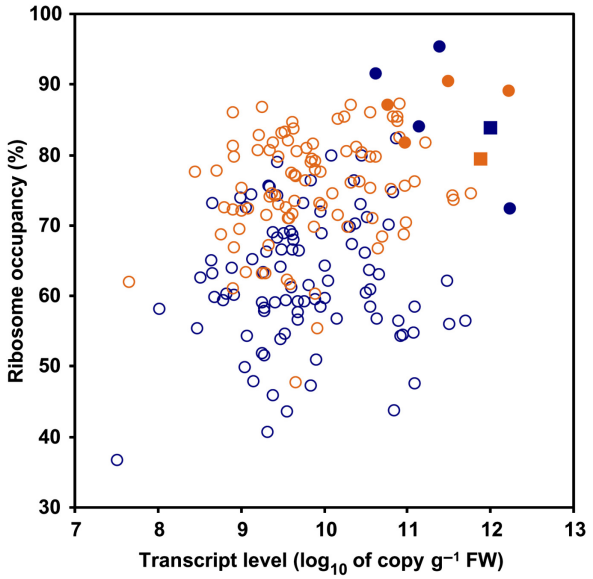

Figure 4 Scatter plot for transcript abundance versus ribosome occupancy in the night and the light period. Ribosomal occupancy was calculated as (SPS + LPS)/(NPS + SPS + LPS). Blue and orange symbols denote plant material collected in the dark and light periods, respectively. Filled symbols denote the *RBCS* gene family (●) and *RBCL* (■). Ribosomal occupancy is weakly, but significantly dependent on transcript concentration in the dark period (Pearson's R^2=0.065, *P*-value=0.011) and light period (Pearson's R^2=0.102, *P*-value=0.001). The plot is generated by using the data provided in Supplementary Table II.

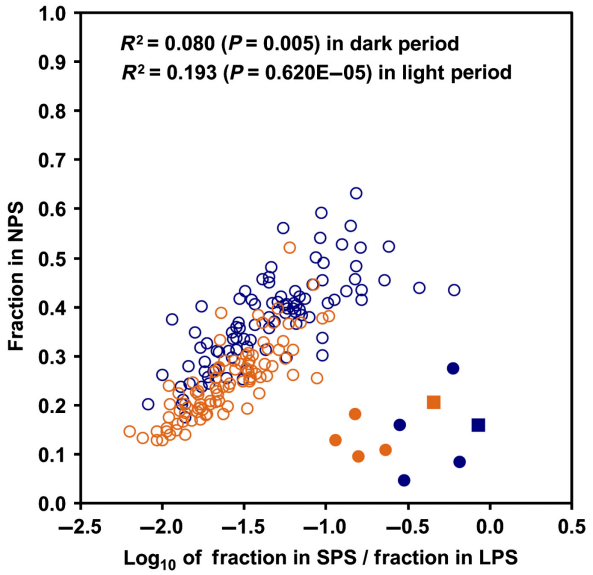

Figure 5 Relation between the fraction of transcript in the non-polysomal fraction (NPS) and the distribution of transcript between the small (SPS) and large polysomal (LPS) fractions. Blue and orange symbols denote plant material collected in the dark and light periods, respectively. Filled symbols denote the *RBCS* gene family (●) and *RBCL* (■). The plot is generated using data provided in Supplementary Table II.

initiation and ribosome progression are determined in a similar manner for all these transcripts. It is consistent with initiation being the limiting step; an increased probability of initiation will result in a decreased fraction of the transcript in the NPS fraction, and will also result in an increased average density of ribosomes per transcript, resulting in a decrease of the proportion found in the SPS compared with the LPS fraction. However, the amount of transcript in the NPS fraction is higher than would be expected from a simple binomial distribution (data not shown); indicating that the probability that ribosomes are recruited to a free transcript is lower than for a transcript that already has bound ribosomes.

The five solid symbols in Figures 3–5 depict the response of *RBCS-1A*, *RBCS-1B*, *RBCS-2B*, *RBCS-3B* (the small nuclear-encoded subunit of RubisCO) and *RBCL* (the large plastid-encoded subunit of RubisCO). These five transcripts deviate strongly from the general relationship between NPS and SPS/LPS. These five transcripts show a high occupancy in the dark as well as the light period, and a large proportion of the transcript is present in the SPS fraction (18–39% and 9–25% in the dark period and light period, respectively) (Figure 5). This could indicate that *RBCL* and *RBCS* transcripts are subject to complex translational control (see Discussion section).

Estimation of translation rates

The rate of synthesis of the proteins encoded by these 98 transcripts was estimated from the transcript abundance in the SPS and LPS fractions, multiplied by the ribosome density per translating transcript (see Calculations and assumptions section, and Supplementary Table II). The calculation assumes an elongation rate of 3 amino acids per ribosome per s, an average of 3 ribosomes per transcript in the SPS fraction, and a ribosome density of 6.6 ribosomes per kb coding sequence, (Brandt *et al*, 2009) in the LPS fraction.

The estimated rates of protein synthesis (mol protein g^{-1} FW h^{-1}) ranged from 2.5E−15 to 2.9E−09 in the dark period, and 6.5E−15 to 4.3E−10 in the light period (Table II and Supplementary Table II). Among the enzymes involved in primary metabolism, RubisCO is the most rapidly synthesized enzyme, reflecting the high abundance of this enzyme in leaves (see Introduction section). Other rapidly synthesized enzymes include several Calvin cycle enzymes (e.g. aldolase, NADP–GAPDH, PGK and TPI), enzymes involved in nitrogen assimilation (e.g. AlaAT, NR and GS), and NAD–GAPDH and NAD–MDH. The relatively high rates of synthesis of PEP carboxylase, NADP–IDH, aconitase and PK compared with other glycolytic enzymes may reflect the fact that these enzymes are required to synthesize 2-oxoglutarate, which acts as the C acceptor during nitrate and ammonium assimilation. The relatively high rate of synthesis of glycerate kinase may be related to the fact that in leaves this enzyme is required for photorespiration. Fluxes through this pathway are roughly 15–20% of those through photosynthesis (Zhu *et al*, 2007), and much higher than in respiratory metabolism. Most enzymes showed an estimated 50–100% increase in the rate of synthesis in the light period compared to the dark period. To further interpret the biological significance of these rates of synthesis, we compared them with the estimated amount of each enzyme in the rosette.

Estimation of protein abundance

Protein abundance of metabolic enzymes in rosette leaves was estimated by two independent methods. In one approach,

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