

# Diurnal and Circadian Light-Harvesting Complex and Quinone B-Binding Protein Synthesis in Leaves of Tomato (*Lycopersicon esculentum*)<sup>1</sup>

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## ABSTRACT

In leaves of tomato (*Lycopersicon esculentum*), the synthesis of a light-harvesting complex (LHC) polypeptide of photosystem II and the quinone B (Q<sub>B</sub>)-binding protein varies at different time points during the day. In vivo labeling with [<sup>35</sup>S]methionine revealed diurnal oscillations of synthesis of these thylakoid membrane proteins. Both proteins are synthesized at elevated levels right after the transition from darkness to light, a maximum is reached around noon, and decreasing levels were measured during the afternoon and night. In addition, in constant darkness both proteins were also synthesized to varied extents at different diurnal time points. Together, these results indicate that the synthesis of a LHC II and the Q<sub>B</sub>-binding protein is under the control of the circadian clock. This circadian oscillation of LHC II protein synthesis correlates with the very well documented circadian *Lhc a/b* mRNA accumulation.

Several proteins and protein complexes of the thylakoid membranes are involved in the first sequence of events of photosynthetic reactions. It is generally assumed that a balanced stoichiometric relationship of these proteins or protein complexes guarantees optimal light harvesting, light conversion, and electron transfer to supply the plant with NADPH (2). The optimized thylakoid membrane composition is challenged when proteins or protein complexes are degraded, which may be due to the specific half-lives of the proteins or to external stress factors such as high irradiance, high temperatures, freezing temperatures, etc. (5, 8, 18–20). There are two alternative ways for the plant to regain photosynthetic function. It can either destroy the other components of the complex and synthesize and reassemble all the subunits again to a new functional complex, or remove and replace only the degraded protein(s) and reuse the other subunits. Clearly, the latter is the most economic strategy (3). However, at present not much is known about the mechanisms that control the amount and timing of synthesis of respective polypeptides in leaves of young or adult plants. As a first step to elucidate this problem, we determined the daily new synthesis of thylakoid membrane proteins in young tomato (*Lycopersicon esculentum*) plants that grew under normal light/dark conditions.

Two important representatives of thylakoid membrane proteins will be discussed in this paper, the LHC<sup>2</sup> proteins and the Q<sub>B</sub>-binding protein. The latter is an important component of the reaction center of PSII. A considerable amount of research has been focused on the remarkably rapid (half-life can be as short as 60 min) and light-dependent turnover of this protein (reviewed in refs. 3 and 15). This polypeptide is encoded in the chloroplast genome by the *psbA* gene, and it has been known for many years that the mRNA produced by this gene is present in greater abundance than the transcripts of any other chloroplast gene. Several stimuli are known to influence the expression and accumulation of the *psbA* mRNA (17).

The other important thylakoid membrane protein or proteins discussed here are those that accomplish the harvesting, transfer, and conversion of the light energy. Several proteins that are structurally and functionally related are known in higher plants that form the LHCs (4, 9, 11). The diversity of respective proteins is genetically determined, e.g. in *L. esculentum* 19 *Lhc a/b* genes so far have been isolated, characterized, and classified with respect to their sequences and intron characteristics (9). A number of previous publications report on several endogenous (developmental stage, organ-specificity, hormones, circadian rhythm [7, 13, 14, 21, 24]) and exogenous (light, temperature [14, 22]) stimuli that influence the expression of the respective *Lhc a/b* genes.

Our particular interest in the past was focused on the circadian rhythm controlling the accumulation of *Lhc a/b* transcripts. Although *Lhc a/b* mRNAs exhibit diurnal and circadian oscillations, neither diurnal steady-state changes at the protein level (21) nor circadian variations in the Chl content or ratio were detectable (16). This discrepancy led us to investigate the diurnal and circadian synthesis of the LHC proteins. In addition, we investigated the diurnal synthesis of the Q<sub>B</sub>-binding protein because the transcription of the *psbA* gene is apparently not under the control of a circadian rhythm (16). The rate of synthesis of these proteins at different time points during the day was determined by labeling young tomato plants in vivo with [<sup>35</sup>S]methionine for 2 h. Newly synthesized proteins at respective diurnal time points were analyzed on SDS-polyacrylamide gels and autoradi-

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<sup>2</sup>Abbreviations: LHC, light harvesting complex; Q<sub>B</sub>, quinone B; LD, light/dark conditions; *Lhc a/b*, (previously *cab*) genes encoding LHC proteins (new nomenclature see ref. 12).

ograms. The results show diurnal and circadian variations in the rate of LHC polypeptide and  $Q_B$ -binding protein synthesis in leaves of young tomato plants.

## MATERIALS AND METHODS

### Plant Material

Tomato seeds (*Lycopersicon esculentum* cv VFNT LA 1221, cherry line) were germinated on moist Whatman No. 3MM paper in continuous darkness. The young tomato plants were transferred into vials and grown hydroponically on Hoagland nutrient solution. The plants were kept under a 16 h/8 h light/dark regimen (light, 6 AM-10 PM) and at temperatures between 20 and 25°C (greenhouse). After approximately 3 weeks, the plants were used for the *in vivo* labeling experiments. Alternatively, plants were kept in continuous darkness (growth chamber) for 1 or 3 d prior to application of [ $^{35}$ S]-methionine or plants were kept in continuous light (growth chamber, 24°C) and were labeled at the 3rd d in continuous light.

### Radioactive Labeling of Proteins

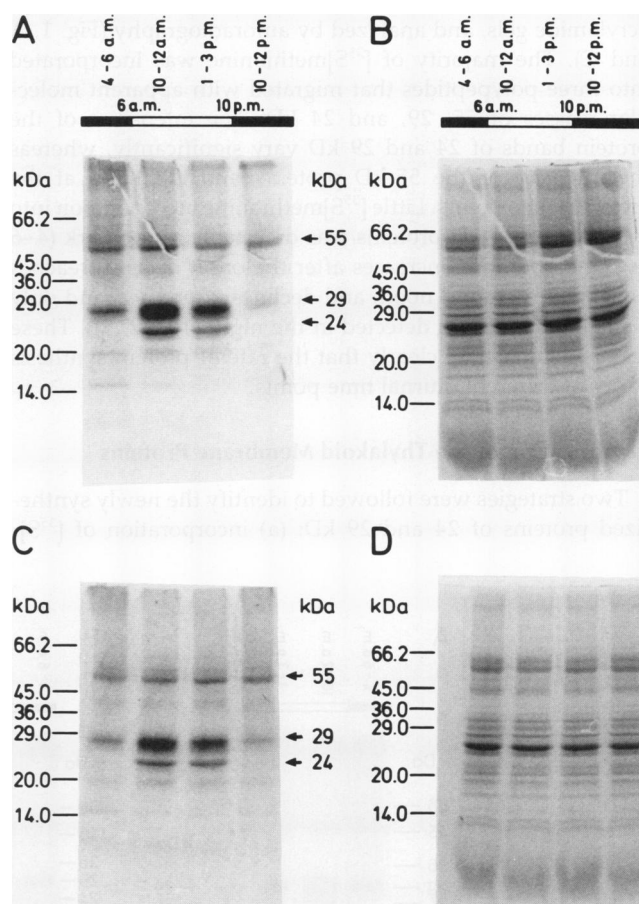
The roots of four young tomato plants were cut off at appropriate time points while the stems were kept submersed. The bases of the cut stems were immediately transferred into 800  $\mu$ L of water containing 50  $\mu$ Ci of [ $^{35}$ S]-methionine (1000 Ci/mmol) and labeled for 2 h. In two series of experiments, either cycloheximide (20  $\mu$ g/mL) or chloramphenicol (400  $\mu$ g/mL) was added to the water/methionine solution. At the end of each labeling process, leaves were harvested, immediately frozen in liquid nitrogen, and stored at  $-40^\circ\text{C}$  until further analysis.

### Isolation of Thylakoid Membrane Proteins

Isolation of thylakoid membrane proteins was performed as described by Steinback et al. (23). Some modifications were made. Three to four grams of leaf tissue was ground in liquid nitrogen. The fine powder was thawed in 8 mL of isolation buffer (50 mM Tricine-NaOH, pH 7.8, 0.4 M sorbitol), filtered through two and eight layers of cheesecloth, and centrifuged for 10 min at 1000g. Proteins of the soluble fraction were precipitated with 70%  $(\text{NH}_4)_2\text{SO}_4$ , resuspended in 10 mM Tricine, 10 mM NaCl, dialyzed against the same buffer for 4 h, and stored at  $-80^\circ\text{C}$ . The membrane pellet was resuspended in 4 mL of buffer (100 mM sorbitol, 5 mM  $\text{Na}_2\text{EDTA}$ , pH 7.8), centrifuged for 5 min at 10,000g, resuspended in 4 mL of 100 mM sorbitol, and centrifuged again for 5 min at 10,000g. The resulting pellet was resuspended in distilled water to a final concentration of 0.8 mg of Chl/mL. Proteins were solubilized with 0.8% Triton X-100 (w/v), gently stirred in the dark at room temperature for 30 min, and centrifuged at 40,000g for 30 min. The extracted thylakoid membrane proteins were frozen and stored at  $-80^\circ\text{C}$ .

The thylakoid membrane proteins were analyzed on SDS-polyacrylamide gradient gels (10–20%). Radioactivity of the protein samples were determined by precipitation of aliquots in 10% TCA, resuspension in Laemmli buffer, and transfer into scintillation mixture (Emulsifier Safe, Packard). To allow

direct comparison of different protein preparations, the same amount of radioactivity was applied per lane. The specific activity (cpm/ $\mu$ g of protein; indicated in the figure legends) is virtually the same during the day; therefore, the same results are obtained when equal cpm/lane (Fig. 1, A and B) or equal  $\mu$ g/lane (Fig. 1, C and D) were applied. Changes in protein synthesis, which occur at different time points, are indicated by different distributions of equal amounts of radioactivity into newly synthesized proteins. Prior to the application on the SDS-polyacrylamide gels, the proteins were solubilized in Laemmli sample buffer and denatured at  $100^\circ\text{C}$  for 2 min. Proteins were stained with 0.1% (w/v) Coomassie brilliant blue R (methanol:acetic acid:water, 4:1:5). Polypeptides labeled with [ $^{35}$ S]-methionine were detected by fluorography: destained SDS-polyacrylamide gels were impregnated



**Figure 1.** Diurnal synthesis of thylakoid membrane proteins. Young tomato plants were hydroponically grown for 3 weeks under day/night conditions (day and night times are indicated by open and filled bars). At indicated time points, four plants were de-rooted and labeled with [ $^{35}$ S]-methionine for 2 h. Thylakoid membrane proteins were isolated, separated on an SDS-polyacrylamide gradient gel (B, 10,000 cpm/lane; D, 120  $\mu$ g/lane; specific activities at 4–6 AM, 10–12 AM, 1–3 PM, and 10–12 PM were 60, 64, 59, 62 cpm/ $\mu$ g protein), and labeled proteins were visualized by fluorography (A and C; exposure time, 21 d). Three highly labeled protein bands of 55, 29, and 24 kDa are indicated by arrows. The latter two accumulate to different extents at different diurnal time points.

with Amplify (Amersham Buchler, Braunschweig, Germany) for 30 min, dried, and exposed to x-ray films at  $-80^{\circ}\text{C}$ .

LHC proteins and the  $\text{Q}_\text{B}$ -binding protein were identified by antibodies raised against LHC II proteins of pea (a generous gift of Neil Hoffman) or antibodies raised against the quinone-binding protein of maize (a generous gift of Lee McIntosh).

## RESULTS

### Diurnal Protein Synthesis

Tomato plants were grown for 3 weeks under day/night (light/dark) cycles with lights on between 6 AM and 10 PM. The synthesis of thylakoid membrane proteins was analyzed at four diurnal time points. At 4 AM, 10 AM, 1 PM, and 10 PM, four plants were derooted and incubated with [ $^{35}\text{S}$ ]methionine for 2 h. At the end of the labeling process, thylakoid membrane proteins were extracted, separated on SDS-polyacrylamide gels, and analyzed by autoradiography (Fig. 1, A and C). The majority of [ $^{35}\text{S}$ ]methionine was incorporated into three polypeptides that migrated with apparent molecular masses of 55, 29, and 24 kD. The intensities of the protein bands of 24 and 29 kD vary significantly, whereas the intensity of the 55-kD protein band is similar at the different time points. Little [ $^{35}\text{S}$ ]methionine incorporation into the 29- and 24-kD proteins was detectable in the dark (4–6 AM). Incorporation increases after the onset of light, reaches a maximum around noon, and declines after 1 PM, and only a small amount was detected in the night (10–12 PM). These results demonstrate clearly that the rate of protein synthesis varies at different diurnal time points.

### Identification of the Thylakoid Membrane Proteins

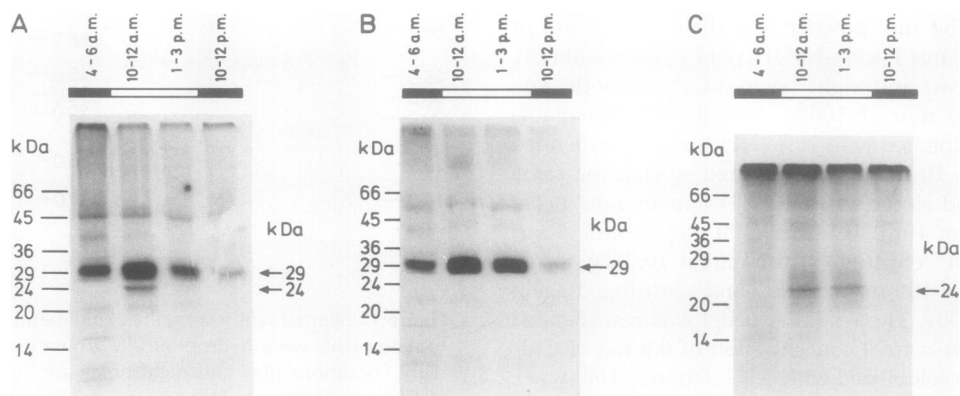
Two strategies were followed to identify the newly synthesized proteins of 24 and 29 kD: (a) incorporation of [ $^{35}\text{S}$ ]-

methionine during application of antibiotics (Fig. 2); and (b) detection with specific antibodies (Fig. 3).

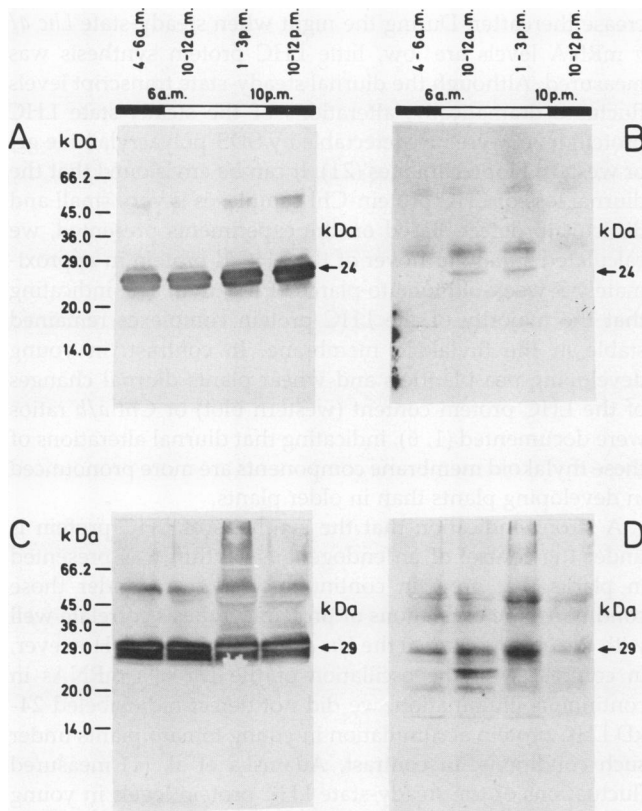
Young tomato plants were simultaneously incubated in [ $^{35}\text{S}$ ]methionine without the addition of antibiotics (Fig. 2A) or with cycloheximide (Fig. 2B) or chloramphenicol (Fig. 2C). Similarly as in Figure 1, the 29- and 24-kD thylakoid membrane proteins show different diurnal accumulation in the control experiment (Fig. 2A). After addition of cycloheximide, only the 29-kD protein and not the 24-kD protein band was detectable (Fig. 2B). In contrast, after the addition of chloramphenicol the 24-kD protein band was evident but the 29-kD band was absent (Fig. 2C). This result suggests that the 29-kD protein is synthesized by the plastid translation machinery, but the 24-kD protein is synthesized on cytoplasmic ribosomes. It is concluded that the 29-kD protein is encoded by the plastid genome and the 24-kD protein by the nuclear genome.

Further identification was obtained through the binding of antibodies (western blots, Fig. 3). Specific antibodies against the LHC proteins of PSII detected primarily a protein band of 24 kD, as well as two minor protein bands of 22 and 26 kD (Fig. 3A). For direct comparison, an x-ray film was exposed to the same blot and the newly synthesized thylakoid membrane proteins became visible (Fig. 3B). It is the labeled 24-kD protein band that was identified by the antibody specific against the LHC protein of PSII, whereas the antibody did not cross-react with the dominantly labeled 29-kD protein band, indicating that these proteins do not share similar epitopes and supporting the notion that they are structurally unrelated proteins. In contrast, the antibody specific against the  $\text{Q}_\text{B}$ -binding protein identified the 29-kD protein of the tomato thylakoid membrane preparation but did not react with the 24-kD protein (Fig. 3, C and D).

These experiments, the apparent molecular mass (Fig. 1), and the fact that the proteins are not present in the soluble fraction (Fig. 4) suggest that the 24-kD protein band repre-



**Figure 2.** Protein synthesis after application of chloramphenicol or cycloheximide. Tomato plants were grown as described in Figure 1. Light and dark phases are indicated by open and filled bars. At indicated time points, four derooted plants were transferred into the labeling mixture, additionally containing either no antibiotic (A), 20  $\mu\text{g}/\text{mL}$  cycloheximide (B), or 400  $\mu\text{g}/\text{mL}$  chloramphenicol (C). Thylakoid membranes were isolated and separated on SDS gels (A and B, 10,000 cpm/lane; C, 5,000 cpm/lane; specific activities at 4–6 AM, 10–12 AM, 1–3 PM, 10–12 PM in panel A were 140, 169, 140, 100 cpm/ $\mu\text{g}$  protein; in panel B, 105, 370, 218, 97, cpm/ $\mu\text{g}$  protein; and in panel C, 54, 46, 48, 42 cpm/ $\mu\text{g}$  protein; exposure time, 4 weeks). The diurnal synthesis of a 29-kD plastid-encoded protein (B) and 24-kD nuclear-encoded protein (C) are indicated by arrows.



**Figure 3.** Identification of proteins by specific antibodies. Thylakoid membrane proteins were separated on SDS-polyacrylamide gradient gels and transferred to nitrocellulose membranes (western blotting). A and B, 4000 cpm per lane; specific activities at 4–6 AM, 10–12 AM, 1–3 PM, 10–12 PM were 61, 64, 60, and 63 cpm/ $\mu$ g protein; exposure time, 6 weeks. C and D, 8000 cpm per lane; specific activities at 4–6 AM, 10–12 AM, 1–3 PM and 10–12 PM were 140, 169, 140, and 100 cpm/ $\mu$ g protein; exposure time, 4 weeks. Proteins were identified by antibodies against the LHC II proteins (A) or against the  $Q_B$ -binding protein (C). Blots were then autoradiographed to illustrate the corresponding labeled thylakoid membrane proteins (B and D, respectively).

sents a tomato LHC protein of PSII, and the 29-kD protein is most likely identical with the  $Q_B$ -binding protein of PSII (32-kD protein).

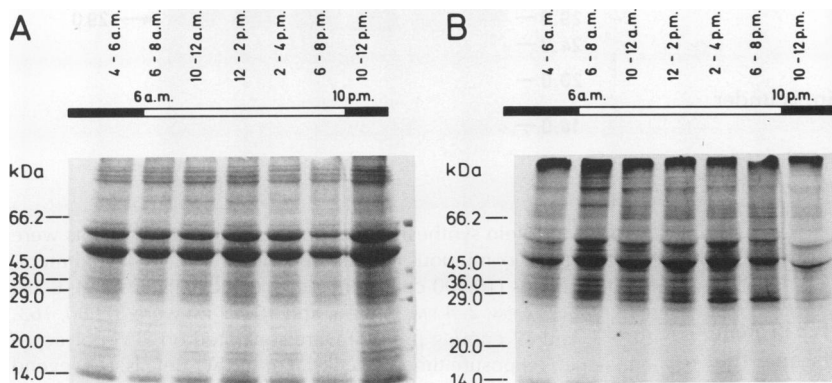
### Protein Synthesis under Constant Conditions

We examined whether the diurnal synthesis of the thylakoid membrane proteins may be due to a circadian rhythm. For this purpose, the tomato plants were grown without a change of the environmental conditions, and they were either transferred into continuous darkness (Fig. 5, A and B) or into continuous illumination (Fig. 6).

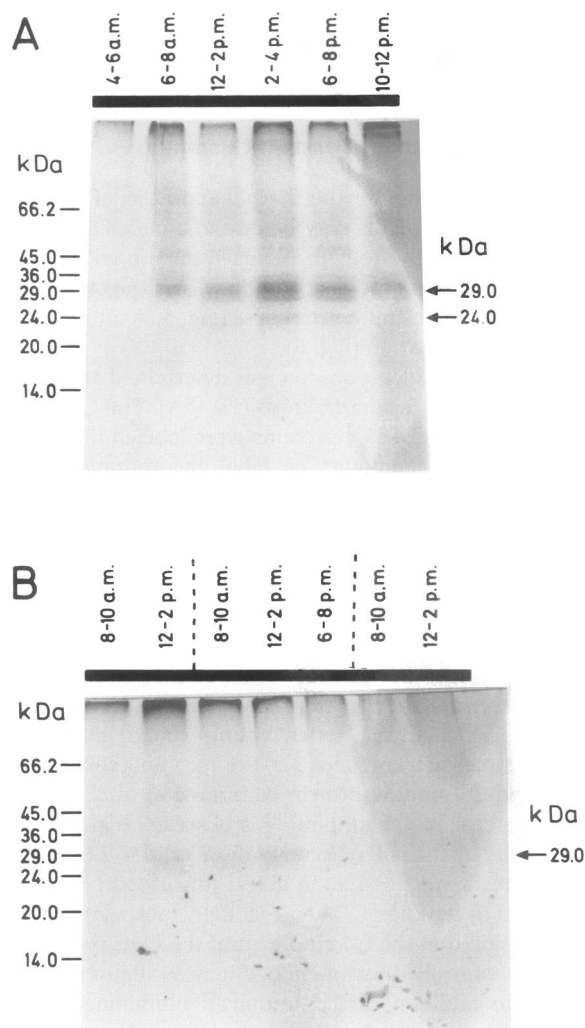
$[^{35}\text{S}]$ Methionine incorporation was determined at indicated time points during 1 d in darkness (Fig. 5A). The  $Q_B$ -binding (29 kD) and LHC (24 kD) proteins were labeled in complete darkness and the amount of label incorporation varied throughout the 24-h period. The levels of both polypeptides increase between 4 AM and 2 PM, reaching a maximum at 2 PM, and amounts decrease thereafter. This variation suggests that the synthesis of these two thylakoid membrane proteins is under the control of a circadian rhythm. It should be pointed out that the time point of maximum accumulation of both polypeptides is delayed by about 2 h in comparison with the accumulation pattern observed under light/dark conditions. Furthermore, when young tomato plants were kept in complete darkness for 3 d, the accumulation of newly synthesized  $Q_B$ -binding protein continued to oscillate; however, a decrease of the amplitude is observed (Fig. 5B). Due to the generally smaller accumulation of the 24-kD LHC polypeptide, it was possible to detect this protein band only at the 1st d in darkness. Taken together, these data support the conclusion that the  $Q_B$ -binding and the LHC proteins are most likely controlled by an endogenous oscillator (circadian rhythm). In addition, in continuously illuminated tomato plants only minor diurnal fluctuation of newly synthesized  $Q_B$ -binding protein was observed, and the 24-kD LHC protein band was not detectable under these conditions (Fig. 6).

### DISCUSSION

To our knowledge, this is the first demonstration that the *in vivo* synthesis of two thylakoid membrane polypeptides,



**Figure 4.** Soluble protein fraction. Tomato plants were grown as described in the legend of Figure 1. Light and dark phases are indicated by open and filled bars. At indicated time points, plants were transferred into the labeling mixture for 2 h. Soluble proteins were isolated and separated on an SDS gradient gel (A), and labeled proteins were visualized by fluorography (B) (40,000 cpm per lane; specific activities at 4–6 AM, 6–8 AM, 10–12 AM, 12–2 PM, 2–4 PM, 6–8 PM, and 10–12 PM were 1190, 1923, 1617, 1097, 1583, 1739, and 726 cpm/ $\mu$ g protein; exposure time, 15 d). Accumulation of LHC and  $Q_B$ -binding proteins was not detectable.



**Figure 5.** Protein synthesis in continuous darkness. Young tomato plants were grown hydroponically for 3 weeks and then transferred to continuous darkness for 24 h (A) or 72 h (B). Thylakoid membrane proteins (A, 10,000 cpm per lane; specific activities at 4–6 AM, 6–8 AM, 12–2 PM, 2–4 PM, 6–8 PM, and 10–12 PM were 26, 47, 55, 86, 99, 70 cpm/ $\mu$ g protein. B, 7,000 cpm per lane; specific activities at 8–10 AM, 12–2 PM, 8–10 AM, 12–2 PM, 6–8 PM, 8–10 AM, and 12–2 PM were 29, 72, 83, 126, 86, 60, 62 cpm/ $\mu$ g protein) were separated on SDS-polyacrylamide gradient gels and visualized by fluorography (exposure times: A, 3 weeks; B, 4 weeks). The synthesis of the 24- and 29-kD protein under respective conditions is indicated by arrows. Light and dark phases are indicated by open and filled bars.

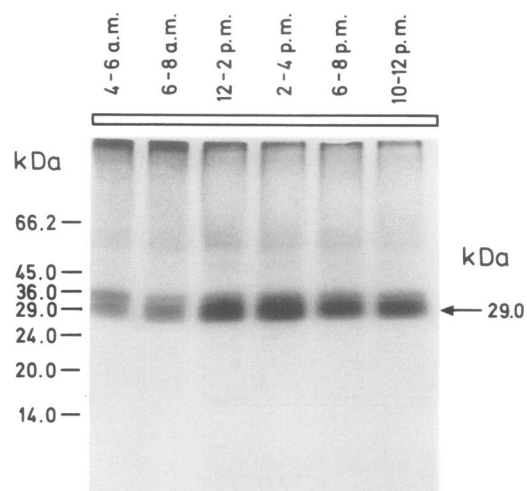
an LHC protein of PSII and the  $Q_B$ -binding protein, is under the control of a circadian rhythm.

The finding of diurnal new synthesis of LHC proteins in young tomato plants correlates very well with the circadian oscillations of the *Lhc a/b* mRNA levels that have previously been documented in tomato and several other plant species (21, reviewed in 13). In tomato leaves, the *Lhc a/b* mRNAs increase dramatically after the onset of light, which coincides with the substantial increase of the 24-kD LHC protein synthesis presented here. Both the mRNA levels and the protein synthesis reach a maximum around noon and de-

crease thereafter. During the night when steady-state *Lhc a/b* mRNA levels are low, little LHC protein synthesis was measured. Although the diurnal steady-state transcript levels fluctuate dramatically, alterations of the steady-state LHC protein level were not detectable by SDS-polyacrylamide gel or western blot techniques (21). It can be envisioned that the diurnal loss of LHC protein-Chl complexes is very small and difficult to detect. Based on the experiments presented, we calculated a daily turnover of the LHC II protein in approximately 3-week-old tomato plants of less than 1%, indicating that the majority of the LHC protein complexes remained stable in the thylakoid membrane. In contrast, in young developing pea plantlets and wheat plants diurnal changes of the LHC protein content (western blot) or Chl *a/b* ratios were documented (1, 6), indicating that diurnal alterations of these thylakoid membrane components are more pronounced in developing plants than in older plants.

A strong indication that the synthesis of LHC protein is under the control of an endogenous rhythm was presented in plants that grew in continuous darkness. Under those conditions, the oscillations of protein synthesis correlate well with the observations at the *Lhc a/b* transcript level. However, in contrast with the oscillation of the *Lhc a/b* mRNAs in continuous illumination, we did not detect radiolabeled 24-kD LHC protein accumulation in young tomato plants under such conditions. In contrast, Adamska et al. (1) measured fluctuations of the steady-state LHC protein levels in young pea plantlets kept under continuous light. The respective data reveal that some very minor fluctuations of the LHC protein amounts are present; however, the lengths of the periods vary significantly. This does not coincide with the almost constant period lengths of true circadian rhythms.

In addition to the LHC protein, another prominent thylakoid membrane protein, the  $Q_B$ -binding protein, was inves-



**Figure 6.** Protein synthesis in continuous illumination. Plants were transferred to continuous light conditions for 1 d. Thylakoid membrane proteins (10,000 cpm per lane; specific activities at 4–6 AM, 6–8 AM, 12–2 PM, 2–4 PM, 6–8 PM, and 10–12 PM were 57, 80, 163, 86, 77, and 77 cpm/ $\mu$ g protein) were separated on SDS-polyacrylamide gels (exposure time, 3 weeks). For further details, see legend to Figure 5.

tigated. To our surprise, we observed diurnal and circadian alterations of protein synthesis. Because no oscillations of the *psbA* mRNA levels were detectable in previous experiments, the circadian synthesis of the respective protein seems to be a contradiction (16). However, the *psbA* mRNA levels are very abundant compared with other plastid or nuclear-encoded genes. It is likely that a small portion of the *psbA* transcripts is used for the diurnal and circadian synthesis of the  $Q_B$ -binding protein, but no changes are detectable when steady-state *psbA* transcript levels are measured by northern blot analysis.

The picture that emerges from the data presented suggests that the thylakoid membrane proteins that degrade due to their half-life times or to various influences during the light period and/or during the night of the previous day have to be substituted at the very beginning of the light period of the following day and that this substitution reaction appears to be under the control of an endogenous rhythm. This mechanism(s) enables the restoration of the thylakoid membrane protein complexes very early in the morning to allow optimal photosynthetic reactions during the upcoming day. This conclusion is in agreement with the fact that the last step in Chl synthesis, the conversion of Pchl<sub>ide</sub> to Chlide, is strictly light dependent (10), suggesting that Chl synthesis occurs right after sunrise, accompanying the thylakoid membrane protein synthesis.

However, virtually nothing is known about the mechanism(s) (transduction chain, involvement of circadian clock, influence of physiological changes, limiting steps, etc.) that lead to such LHC- and  $Q_B$ -binding protein fluctuations. At the present stage of knowledge, it seems unlikely that the uptake of the cytoplasmic LHC precursor is a limiting step, because the precursor does not accumulate in the soluble fraction (Fig. 4, A and B). To understand the regulatory process(es) of diurnal/circadian thylakoid membrane protein synthesis, further investigations are necessary.

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