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Plastid and nuclear mRNA fluctuations in tomato leaves – diurnal and circadian rhythms during extended dark and light periods

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Abstract

Steady-state mRNA levels of nuclear (*rbc*S, *cab, tub*A) and plastid (*rbc*L, *psb*A) encoded genes were determined in tomato leaves of different developmental stages. Transcripts were analyzed at four-hour intervals throughout a diurnal cycle in 4 cm-long terminal leaflets, while mRNA levels of the chlorophyll a/b-binding protein (*cab*), and the small and large subunit of RuBPC/Oase (*rbc*S, *rbc*L) are high. At different time points during the day the mRNAs accumulate to characteristic levels. Minor fluctuations of such mRNA levels were determined in the case of *rbc*S, *rbc*L, *psb*A and *tub*A, while significant alterations are observed for the chlorophyll a/b-binding protein transcript levels. LHCP II transcripts accumulate during the day, reach highest levels at noon and decline to non-detectable levels at 5 a.m. The *cab* mRNA fluctuates with a periodic length of approximately 24 hours suggesting the existence of a circadian rhythm ("biological clock"), which is involved in gene activation and inactivation. The mRNA oscillation with the same periodic length, but altered amplitude, continues to be present in plants which are kept under extended dark or light conditions. Different mRNA fluctuation patterns are observed for *rbc*S, *rbc*L, and *psb*A under such experimental conditions.

Introduction

Plants are complex organisms responding to many different stimuli, some of them affecting gene expression at the molecular level. Mechanisms that regulate gene expression have been studied in detail particularly with photosynthesis-specific plastid and nuclear genes, such as the large and small subunit of ribulose-1,5-bisphosphate carboxylase (RuBPC/Oase), the light harvesting complex proteins (LHCP), and PSI and PSII protein components [3, 7, 18, 19, 21]. The analysis of these genes in normal and transgenic plants demonstrated that gene expression can be controlled or influenced by more than one factor [3, 7, 17, 18]. In general, the activation and inactivation of genes is based on a characteristic developmental program [11, 12]. Additionally, this pattern can be modulated by several superimposed stimuli such as organ- and tissue-specificity, and environmental conditions such as light, temperature, soil composition etc. [7, 17]. A recent detailed investigation of mRNA levels of photosynthesis-specific and non-photosynthesis-specific genes in developing tomato fruits revealed that characteristic alterations occur during a natural day/night cycle [13]. These mRNA fluctuations follow a certain periodicity suggesting that diurnal or circadian rhythms are also involved in controlling and/or regulating the expression of genes at the transcriptional and/or post-transcriptional level. Based

on these results I started to investigate such rhythms in plants in more detail.

Circadian rhythms are endogenously generated biological oscillations with periods of approximately 24 hours. Such rhythms are characterized by the following features: (1) appearance of "free-running" cycles in constant conditions, (2) oscillations are temperature independent in a certain range, and (3) oscillations can be synchronized or entrained by external light/dark cycles [6]. However, so far no single component of the circadian oscillator mechanism has yet been definitely identified in any organism [8]. Moreover, little is known about the molecular basis of circadian rhythms [5, 6, 8].

First indications of the existence of circadian rhythms at the genetic level are supported by nucleus exchange experiments [15], the occurrence of "clock mutants" (for review see ref. [6]), and the periodic mRNA fluctuations of photosynthesis-specific genes during day/night cycles in developing tomato fruits [13]. Based on the data of the latter, two series of experiments were performed to try to obtain some more insight into the mechanisms of the transcript level oscillations in plants. In tomato leaves of plants kept under extended light or dark conditions, mRNA levels of the large and small subunit of RuBPC/Oase, the LHCP, and the Q_B-binding protein were analyzed.

Methods

Plant material and tissue preparation

Tomato plants (*Lycopersicon esculentum,* cv. VFNT LA 1221, cherry line) were grown in greenhouses at the University of Göttingen without additional light and at temperatures between 20–25 °C. Terminal leaflets of different sizes (1.0–1.5 cm; 2 cm; 4 cm; 6 cm) were harvested from 6-week-old vegetative tomato plants, and from 10-week-old plants (6 cm green leaflets of the middle section of the plant and 6 cm yellowish leaves from the lower section of the plant, representing the second oldest and oldest leaves in this series of experiments, respectively). The leaves were harvested at noon 29th May 1987. For the "continuous light/dark" experiments five plants were exposed continuously to light for five days (Osram Power Star HQI-TS 250 W/D), five plants were kept for five days in a dark tent, and two plants under the natural diurnal day/night cycle. Leaflets of all five or two plants were harvested at each timepoint: 5 a.m., noon and 5 p.m. Plants were shifted to the light and dark conditions or back to the natural diurnal day/night cycle between 5 and 6 p.m. At the first day of the experiment sunrise was at 6:31 a.m. and sunset at 6:30 p.m., at the last day of the experiment (8th day) sunrise was at 6:15 and sunset at 6:43. Leaflets were immediately frozen in liquid nitrogen and stored at −80 °C.

RNA isolation and analysis

RNA was isolated from 0.25–1.1 g leaf tissue according to the method described elsewhere [12, 13]. RNA from different preparations were standardized and analyzed by spectrophotometric quantitation, ethidium bromide fluorescence of the cytoplasmic rRNA in stained gels and relative levels of hybridization with heterologous cytoplasmic rDNA. RNA was separated by formaldehyde agarose gel electrophoresis and transferred to nylon filter (Amersham, Hybond N). Specific gene probes for chlorophyll a/b-binding protein (*cab* 1B), small and large subunit of the RuBPC/Oase (*rbc*S2A, *rbc*L), Q_B-binding protein of photosystem II (*psb*A) [12, 13], and alpha subunit of tubulin (*tub*A) were used for hybridization (0.5 kb *Eco*RI-*Bam*HI fragment of the coding region of a pea tubulin A gene).

To determine relative transcript levels of specific mRNAs autoradiograms were scanned with a Joyce Loebl densitometer and mRNA was quantitated by peak area measurements. Relative mRNA levels are based on two or three hybridizations. Steady-state mRNA levels of *cab*, *rbc*L, and tubulin A (Fig. 2) are based on one hybridization.

Results

In a first set of experiments we analyzed steady-state mRNA levels of a variety of plastid- and nuclear encoded genes during leaf development. Figure 1A

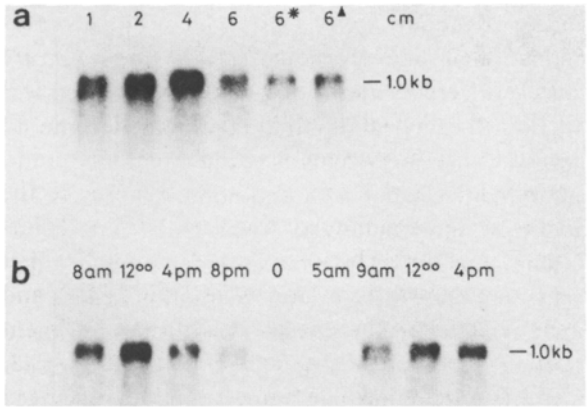

Fig. 1. A representative example of Northern blot analysis of RNA levels in tomato leaves.

A: Total RNA was extracted from terminal leaflets of different length (1 to 6 cm; * leaflets of the middle section of the plant; ▲ leaflets from the lower section of the plant). Leaves were harvested at noon.

B: Total RNA was extracted from 4 cm-long terminal leaflets at four-hour intervals during a diurnal cycle.

RNA was separated on formaldehyde-agarose gels and transferred to nylon filters. Filters were hybridized with a specific probe for the chlorophyll a/b-binding protein (*cab*1B), exposed for six days with intensifying screen at −70 °C. The length of the transcripts is indicated.

presents a typical example of the Northern blot analysis. In a second series of experiments gene expression levels were determined and compared at one developmental stage during a 24-hour period to monitor possible diurnal mRNA fluctuations (Fig. 1B). To evaluate whether light has an influence on diurnal transcript fluctuations, total RNA was isolated from plants, which were kept under continuous light or continuous dark conditions. In this series of experiments the mRNA levels of four photosynthesis-specific genes (*rbc*S, *rbc*L, *cab* and *psb*A) were measured.

mRNA levels during leaf development

Northern blots were prepared with total RNA isolated from leaves of different developmental stages. Terminal leaflets were picked at noon and categorized by their length. Hybridization of the Northern blots with the coding region of *rbc*L and a cDNA of *rbc*S2A reveal the characteristic transcripts of 1.8 kb and 0.9 kb, respectively [12]. Relative transcript levels of the ssu and lsu of RuBPC/Oase in different developmental stages of leaves are shown in Fig. 2A. Both transcripts are present at highest levels in 4 cm-long leaves and decrease continuously during further growth. Significant differences in expression pattern of *rbc*S and *rbc*L of RuBPC/Oase are observed in young tomato leaves. The *rbc*S levels are already high in young leaves, while *rbc*L transcript levels increase continuously during the young stages.

Similar to the mRNA levels of the stromal RuBPC/Oase, the transcript levels of two thylakoid membrane proteins, the nuclear-encoded light-harvesting chlorophyll a/b-binding protein (LHCP II) and the plastid encoded Q_B-binding protein of photosystem II, were tested (Fig. 2A). The mRNA

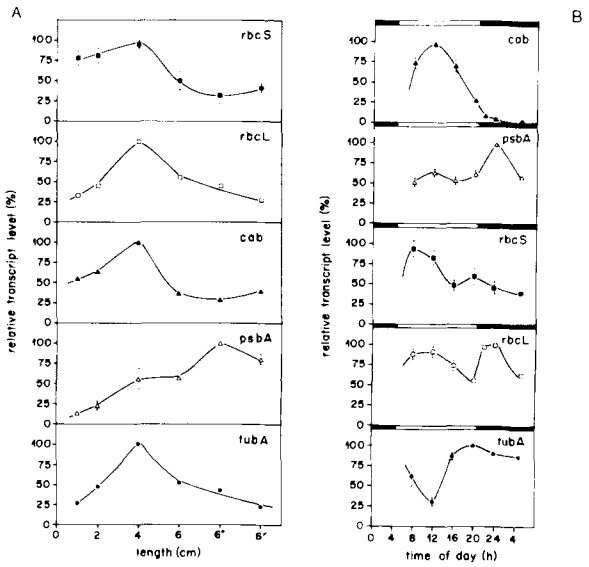

Fig. 2.

A: Relative changes of mRNA levels of the small (*rbc*S, ■) and large (*rbc*L, □) subunit of RuBPC/Oase, the chlorophyll a/b-binding protein (*cab*, ▲), the Q_B-binding protein of PS II (*psb*A, △), and the alpha subunit of tubulin (*tub*A, ●) during tomato leaf development. Relative transcript levels were calculated based on densitometer scannings of an autoradiogram of two gels. Error bars represent ±SE.

B: Relative changes of mRNA levels of the LHCP II (▲), the Q_B-binding protein (△), the small (■) and large (□) subunit of RuBPC/Oase and the alpha subunit of tubulin (●) during a 24 hour period (diurnal cycle). Phases of light and darkness are indicated at the top of each panel by open or filled bars, respectively. Relative transcript levels were calculated based on densitometer scannings of two hybridizations. Error bars represent ±SE.

levels of the LHCP II genes are highest in 4 cm-long leaves, while the transcripts of the plastid-encoded *psb*A gene accumulate to high levels in 6 cm-long leaves. A comparison of the expression pattern of these thylakoid membrane proteins indicates that probably no close coordination exists in gene expression during leaf development.

In addition to photosynthesis-specific genes, the transcript levels of a nuclear-encoded non-photosynthesis-specific gene was determined in tomato leaf extracts (Fig. 2A). A heterologous DNA probe specific for the alpha subunit of tubulin was used for the identification of tomato transcripts. Transcripts of approximately 1.6 kb length were detected in tomato leaf RNA preparations. The tubulin A mRNA accumulates to high levels in 4 cm-long leaves.

Transcript levels in tomato leaves during a diurnal cycle

To characterize possible changes in mRNA levels during a day, 4 cm-long tomato leaves were collected at four-hour intervals over a period of 24 hours. At this developmental stage the mRNA levels are highest for most genes tested in the previous experiment (Fig. 2A).

Northern blots were performed with RNA extracts from six time points during a 24-hour period and hybridized with gene probes specific for thylakoid membrane proteins (*psb*A, *cab*) and relative mRNA concentrations were determined (Fig. 2B). Significant mRNA fluctuations are detected for the LHCP II, while the levels of the Q_B-binding protein are only slightly altered. Highest LHCP II mRNA levels are present at noon, decrease continuously during the afternoon, evening and night, and lowest levels are detected at 5 a.m. in the morning. During the following 7-hour period mRNAs accumulate to their highest levels. These results indicate that LHCP II mRNAs are degraded and newly synthesized over a 24-hour period, suggesting a high turnover rate of less than 12 hours in developing tomato leaves. The experiments demonstrate that mRNA accumulation and degradation follow a certain periodic pattern which might indicate that a diurnal and/or circadian rhythm exists which is involved in regulation of the expression of LHCP genes. In contrast, a different expression pattern is observed for the plastid encoded Q_B-binding protein, accumulation of transcripts occurs between 4 p.m. and midnight and between 8 a.m. and noon. Low levels are detected approximately at 4 p.m. and 5 a.m.

Similar to the thylakoid membrane protein genes the steady-state mRNA level fluctuations of the ssu and lsu of RuBPC/Oase were monitored during a 24-hour period (Fig. 2B). These mRNA fluctuations are only minor in comparison to changes observed for the light harvesting chlorophyll a/b-binding protein mRNAs. The transcripts for the ssu accumulate to high levels approximately at 8 a.m. and decrease to low levels at 5 a.m. A small increase is observed between 4 p.m. and 8 p.m. The transcripts for the lsu of RuBPC/Oase accumulate to high levels twice during a 24-hour period, high levels are detected at noon and at midnight, lowest levels are observed at 8 p.m.

In addition to photosynthesis-specific genes it was of interest whether genes coding for other cellular components are also subject to diurnal mRNA fluctuations. Altered tubulin specific mRNA levels were determined (Fig. 2B). Increasing transcript levels are measured in the afternoon until high levels are reached approximately at 8 p.m. Low mRNA levels are monitored at noon.

Comparison of the fluctuation patterns of the different genes (Fig. 2B) suggests that characteristic diurnal oscillations exist in developing tomato leaves. While the LHCP transcript levels alter dramatically within a 24-hour period, only minor fluctuations were determined for the four other investigated genes. Beside the fluctuation amplitude the time points of high and low mRNA levels during a day characterize the diurnal oscillation pattern. Coincidentally the four photosynthesis-specific mRNAs (*cab, psb*A, *rbc*S and *rbc*L) accumulate to high levels approximately at noon. However, the Q_B-binding protein and the ssu and lsu of RuBPC/Oase have an additional peak at midnight or 8 p.m. The tubulin A-specific transcripts accumulate to high levels approximately at 8 p.m. From these results it appears that two or three different fluctuation patterns are present in tomato leaves.

Determination of mRNA levels in plants grown under continuous light or dark conditions

Leaflets of tomato plants which were grown under either continuous light or dark conditions were harvested at three time points during a day (5 a.m., noon, 5 p.m.). Total RNA was extracted and analyzed by Northern blot analysis using specific hybridization probes, such as LHCP II (*cab*), Q_B-binding protein (*psb*A), and small and large subunit of RuBPC/Oase (*rbc*S, *rbc*L). Relative transcript levels were determined and plotted.

Light harvesting complex proteins of photosystem II (LHCP II)

The analysis of LHCP II mRNA levels in plants kept under continuous light and continuous darkness is shown in Fig. 3. Under such experimental conditions the LHCP-specific mRNA levels continue to fluctuate with the same periodic length as under the natural day/night cycle, high LHCP II mRNA levels are detected at noon, low levels at 5 p.m. and shortly before sunrise (5 a.m.), indicating the existence of a "free-running" rhythm. In continuous illumination (Fig. 3A), the transcript levels at noon are reduced 3–4-fold. This mRNA reduction is apparent, regardless of the length of time the plants are kept under the artificial light conditions (one to five days). When plants are transferred back to the natural day/night cycle accumulation of *cab* transcripts increases to 75% of the normal level in less than one day. It is interesting to note that the mRNA level significantly increases after a night phase of approximately 7 hours. This indicates the necessity of light and dark periods for the optimal or balanced gene expression during plant development.

In continuous darkness (Fig. 3B), the *cab* mRNA levels increase during the morning and decrease in the afternoon and night, similarly to the fluctuation pattern observed under the natural day/night cycle. The mRNA levels oscillate with the same periodicity as under natural conditions. However, in comparison the transcript levels at noon were reduced 3–4-fold one day after plants were shifted into darkness, and the peak of mRNA level at noon decreased continuously the longer the plants stayed in darkness, until after five days no mRNA accumulation could be detected. When plants were shifted back to the natural day/night cycle, transcripts accumulated to approximately 15% of the normal level within a day, oscillating with the characteristic mRNA fluctuation pattern of LHCP. Two days after the transfer of the plants, the peak mRNA level increased 2–3 times and reached approximately 50% of the normal level in leaves.

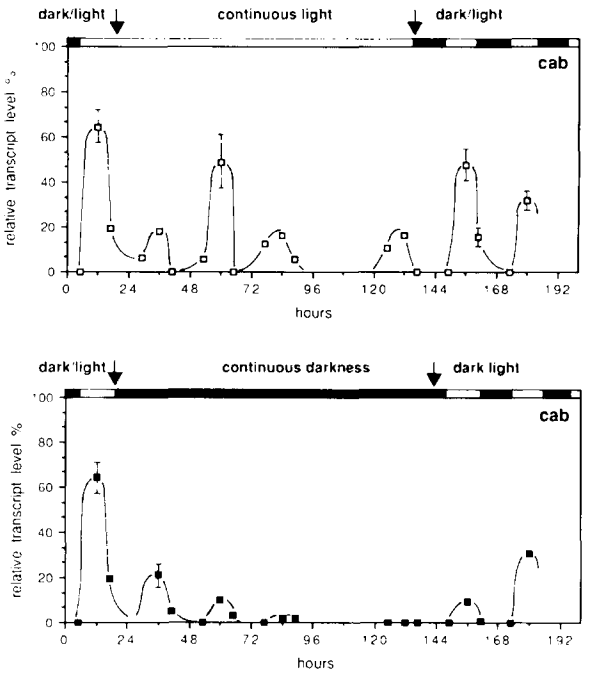

Fig. 3. Relative changes of transcript levels of the chlorophyll a/b-binding protein (*cab*1B) under continuous light conditions (A, □) and continuous dark conditions (B, ■). Phases of light and darkness are indicated at the top of each panel by open or filled bars, respectively. Error bars represent ±SE.

Small and large subunit of RuBPC/Oase

The small and large subunit of RuBPC/Oase mRNAs fluctuate with characteristically small amplitudes within a natural day/night cycle (Fig. 2B). After transferring the plants to continuous light conditions (Fig. 4A, C) mRNA levels of ssu and lsu of RuBPC/Oase in tomato leaves continue to fluctuate for several days. Under such circumstances both mRNAs follow similar oscillation patterns manifested by a comparable amplitude and changes in periodic length. Two and three days after the transfer of the plants the amplitude of both mRNA

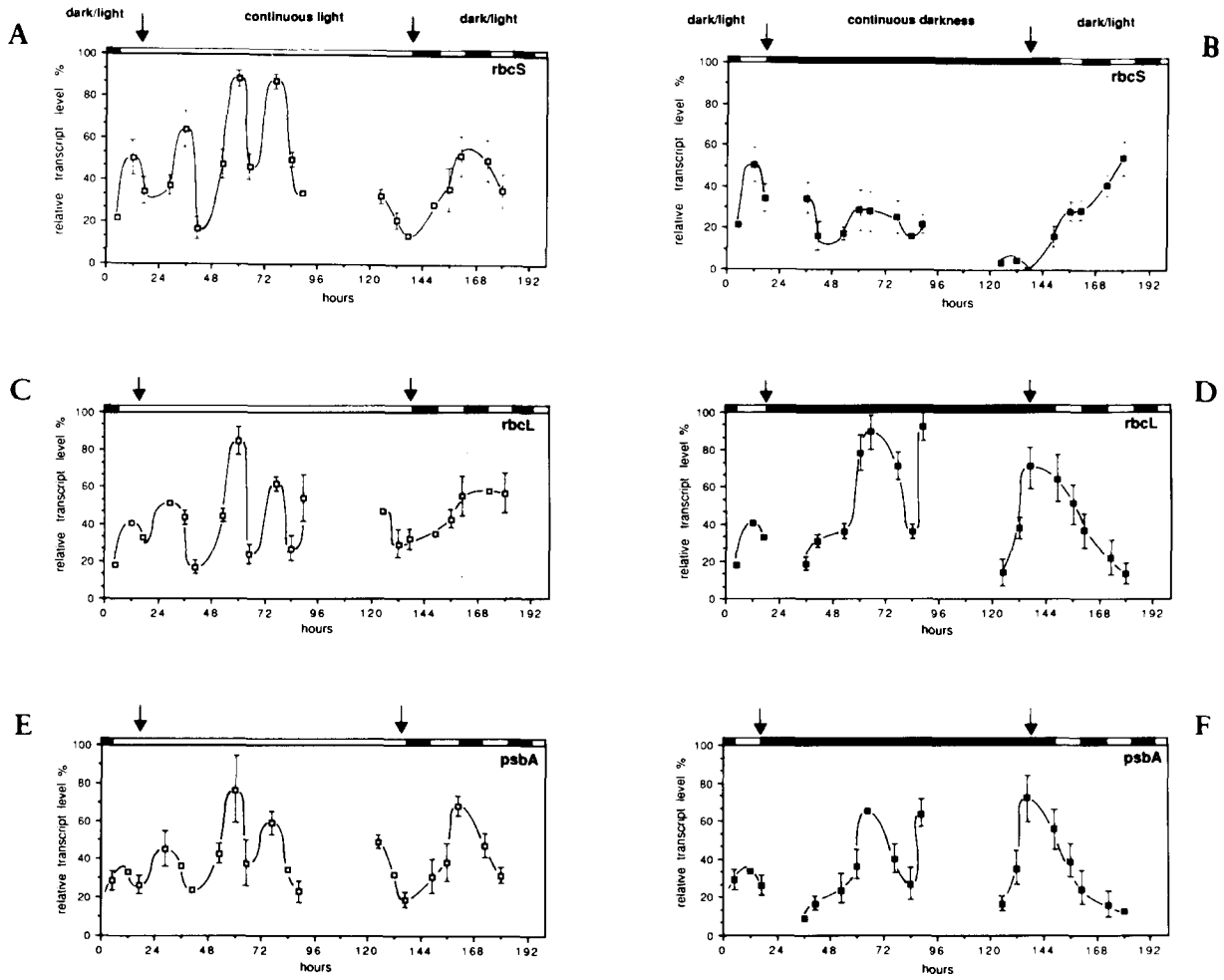

Fig. 4. Relative changes of the small (*rbc*S; A, B) and the large (*rbc*L; C, D) subunit of RuBPC/Oase, and the Q_B-binding protein of PS II (*psb*A; E, F) under continuous light (□) and continuous dark conditions (■). Light and dark phases are indicated at the top of each panel by open or filled bars, respectively. Error bars represent ±SE.

levels are increased 2-fold, while at the fifth day of the experiment the maximum level is less or equivalent to the level measured under normal conditions. When plants are shifted back to the natural day/night situation, both transcripts accumulate synchronously over a period of 24 hours until the steady-state levels start to decrease. However, a reset to the normal mRNA fluctuation pattern is not apparent after two days under natural conditions.

Keeping plants in darkness for several days, the mRNA levels measured for the ssu and lsu vary significantly from the normal mRNA fluctuation pattern (Fig. 4B, D). No periodic pattern can be observed. Only minor fluctuations are determined for the ssu mRNAs, while the transcript concentrations of the lsu in leaves alter as much as 4.5-fold. Interestingly, a peak of high *rbc*L mRNA accumulation two to three days after the shift into darkness is concomitant with increased ssu transcript levels. After five days in darkness only a very small amount of ssu transcript is detectable while the mRNA levels for the lsu of RuBPC/Oase are present and increasing levels are measured from 5 a.m. to 5 p.m. After transferring plants from continuous darkness to the natural day/night cycle, the mRNA levels for the large and small subunit of RuBPC/Oase show opposite

accumulation patterns. Transcript levels for the lsu decrease continuously, while the ssu transcript levels increase continuously, indicating that the oscillation pattern of both subunits of RuBPC/Oase are not synchronized, and a reset to the normal fluctuation does not occur within 2 days of the shift.

Q_B-binding protein of photosystem II

The fluctuation amplitudes for Q_B-specific mRNAs (*psb*A) are only minor compared to those of the LHCP mRNAs in plants grown under natural day/night cycle (Fig. 2B). After shifting plants to constant light conditions mRNA levels vary and some alterations in the periodic length and increased amplitude are detected (Fig. 4E). mRNA level fluctuation remains throughout the five days of the experiment. After shifting back from continuous illumination to natural conditions, *psb*A-specific mRNAs accumulate continuously for 24 hours before the levels start to decline, while after continuous darkness (Fig. 4F) the mRNAs decrease immediately after the shift to the natural cycle. Comparison of the mRNA fluctuation diagrams of the Q_B-binding protein (*psb*A) and the lsu of RuBPC/Oase (*rbc*L) reveals significant homologies suggesting that regulation of plastid gene expression under natural and artificial light/dark conditions seems to be synchronized.

Discussion

Light-harvesting complex proteins

A characteristic diurnal oscillation pattern was monitored for the cab mRNA in tomato leaves; mRNA levels increase during the morning, reach maximum levels approximately at noon, followed by a decline of mRNA accumulation in the afternoon until lowest levels are detected after midnight. Similar fluctuation patterns have been demonstrated in tomato fruits [13], pea and maize seedlings [9], pea leaves [20] and in petunia leaves (P. Dunsmuir and M. Stayton, personal communication), indicating that this gene activation/inactivation mechanism seems to be universal in different tissue types, different organs and different plant species. However, an interesting difference has been noticed. In pea and maize seedlings the LHCP mRNAs are present at elevated levels two hours prior to illumination and do not decrease to undetectable levels during the night [9] as observed in tomato leaves and fruits (Fig. 1B and 2B; ref. [13]). The significance of this discrepancy is yet not understood, but an analysis of several plant species in parallel is under way.

It was of interest whether or not the characteristic alterations of cab mRNA expression are retained under continuous light/dark conditions. The rhythmic pattern with periods of approximately 24 hours continuous to be present for several days under both artificial conditions (Fig. 3), displaying a "free running" cycle. This result suggests that an endogenous – circadian – rhythm is involved in the expression of cab genes in tomato leaves. While the principal periodic pattern is conserved under such constant external conditions, the amplitude of expression levels are altered. In constant darkness the mRNA levels at noon decline continuously to undetectable levels. This damping phenomenon is characteristic for endogenous rhythms, and has been observed with several physiological and morphological oscillations in plants [2]. The periodic pattern can be re-established or re-initiated as soon as the plants are returned to alternating light/darkness conditions.

The following observations additionally support the hypothesis of a circadian rhythm involved in cab gene expression. Decreasing mRNA levels are monitored in the afternoon when plants are grown in a greenhouse or with artificial light in a growth chamber. This result is surprising, since in several plant species it has been demonstrated that light induces cab gene expression [22]. In addition, when plants are grown in continuous light the same oscillation pattern as under the natural day/night conditions is observed. Therefore, even when light is available cab mRNA accumulation declines in the afternoon. These results favor the hypothesis that an endogenous rhythm, superimposed over light induction, controls the expression of LHCP genes in tomato leaves. Presently it is not known how much mRNA each member of the multigene family contributes to the steady-state levels and whether each member follows a similar expression pattern.

When plants are kept in continuous light condi-

tions the amplitudes for LHCP mRNAs are reduced by approximately 60%, while the mRNAs for the Q_B-binding protein of PS II and the ssu and lsu of RuBPC/Oase are increased compared to the levels in plants grown under the natural day/night cycle. Presently it can only be speculated why mRNAs accumulate to altered levels. It has been noticed that leaf colour changes from green to dark blue green and the pH of the leaf extracts become more basic after continuous illumination, indicating that the plants try to compensate the amount of light available for photosynthetic reactions by changing their pigment composition and physiology.

Plastid and nuclear encoded photosynthesis-specific genes

In addition to the nuclear-encoded light-harvesting chl a/b-binding proteins other photosynthesis-specific genes, the large and small subunit of RuBPC/Oase and the Q_B-binding protein of PS II, were investigated in respect to diurnal mRNA fluctuations in tomato leaves. The mRNA levels of *rbc*L, *psb*A and *rbc*S alter approximately 2-fold within a 24-hour period, therefore the fluctuation amplitude is significantly less pronounced than in the case of cab mRNAs. Similarly, a 2-fold variation and a diurnal rhythmic pattern has been described for *cab*, *rbc*S and *rbc*L mRNAs in greening pea [20]. These results suggest that also plastid gene expression may underlie diurnal rhythmicity.

The diurnal oscillation patterns of *psb*A, *rbc*L and *rbc*S in tomato leaves are altered under continuous light/dark conditions: (1) the amplitudes of mRNA level are changed and (2) the periodic length is slightly varied. The resulting fluctuation patterns of these three genes are very similar. The synchronized expression of the two plastid genes (*psb*A and *rbc*L) both under the artificial light/dark situation and after the shift back to the natural day/night cycle suggest that the expression of both genes is regulated by a similar mode. This result coincides with the results obtained for spinach chloroplast gene expression [4]. No synchronized expression is observed for the ssu and lsu of RuBPC/Oase after a prolonged dark period and shift to the natural day/night cycle; while the ssu mRNAs accumulate, the lsu mRNA levels decrease significantly. Similar accumulation patterns of *rbc*S and *rbc*L mRNAs were observed in maize seedlings [14]. These results are interesting since, during tomato fruit development [12], diurnal cycles in tomato fruits [13], as well as in several other plant species, coordinated expression patterns are observed for these counterpartner genes [1, 16].

In conclusion, the data presented in this report demonstrate the existence of circadian rhythms of nuclear- and plastid-encoded photosynthesis- and non-photosynthesis-specific genes operating at the molecular level in tomato leaves. Different genes seem to be regulated by different circadian oscillators. The results demonstrated by Kloppstech [9] and Spiller *et al.* [20] manifest the idea that a mechanism of a "biological clock" functions at the level of transcription and post-transcription, which has been hypothesized by Ehret and Trucco [5]. At present no factor has been isolated which is evidentially involved in the regulation of oscillating patterns. A possible role in controlling the process of the "biological clock" in *Euglena* was proposed for calmodulin [10]. Further experiments and detailed analyses may bring some more insight into the molecular mechanisms of the "biological clock" in plants.

Acknowledgements

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