Planta (1989) 180:5–15

Planta
© Springer-Verlag 1989

Molecular characterization of the diurnal/circadian expression of the chlorophyll a/b-binding proteins in leaves of tomato and other dicotyledonous and monocotyledonous plant species

Hartmut Meyer, Ute Thienel, and Birgit Piechulla*
Institut für Biochemie der Pflanze, Untere Karspüle 2, D-3400 Göttingen, Federal Republic of Germany

Abstract. Diurnal oscillations of steady-state mRNA levels encoding the chlorophyll a/b-binding proteins were monitored in *Lycopersicon esculentum, Glycine max, Phaseolus vulgaris, P. aureus, P. coccineus, Pisum sativum, Sinapis alba, Hordeum vulgare, Triticum aestivum* and *Zea mays*. In these plant species *cab* mRNA accumulation increases and decreases periodically indicating i) that the expression of the genes for chlorophyll a/b-binding proteins (*cab* genes) is controlled by a circadian rhythm, and ii) that the rhythm is widely distributed among monocotyledonous and dicotyledonous plant species. A detailed characterization of the pattern of *cab* mRNA expression in tomato leaves shows that the amplitude of the oscillation is dependent on i) the developmental stage of the leaves, ii) the circadian phase and duration of light and iii) the circadian phase and duration of darkness. In addition to the chlorophyll a/b-binding proteins, genes coding for other cellular functions were examined for cyclic variations of their mRNA levels. The analysis includes genes involved in i) carbon metabolism (e.g. phosphoenolpyruvate carboxylase, pyruvate orthophosphate dikinase, alpha amylase, fructose-1,6-bisphosphate aldolase, triosephosphate isomerase), ii) photosynthesis (large and small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, Q_B-binding protein, reaction-center protein of photosystem I) and iii) other physiological or morphological reactions (e.g. ubiquitin, actin). However, no periodic fluctuation pattern was detected for the mRNA levels of these genes in tomato and maize leaves.

* To whom correspondence should be addressed

Abbreviations: Chl = chlorophyll; LHCP = light-harvesting chlorophyll a/b-binding protein; Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase

Key words: Chlorophyll a/b-binding protein – Circadian rhythm – *Lycopersicon* (chlorophyll a/b-binding protein, rhythm) – Rhythm (chlorophyll a/b-binding protein)

Introduction

Reports on the phenomenon of circadian clocks have appeared in the literature since about 1900. It is well established that diurnal and/or circadian rhythms are present in all eukaryotic organisms (Feldman 1982). In higher plants, several biochemical, morphological and physiological oscillations are the result of endogenous rhythms (Vince-Prue 1983; Jerebzoff 1986). Examples of the circadian rhythms in plants are the nyctinastic motion of leaves in *Phaseolus* and certain other legumes (Bünning 1956; Satter and Galston 1981) or the diurnal motions of the petals of *Kalanchoe blossfeldiana* (Bünsow 1953). Recently, it was demonstrated that the expression of the genes for the chlorophyll (Chl) a/b-binding proteins (*cab* genes) in tomato fruits and leaves (Piechulla and Gruissem 1987; Piechulla 1988), in pea leaves (Kloppstech 1985) and in wheat leaves (Nagy et al. 1988) are controlled by a biological clock, which is manifested at the transcriptional or post-transcriptional level. Evidence for the existence of circadian clocks at the genetic level was provided by investigations of so-called clock-mutants, e.g. *Acetabularia* (Schweiger 1969), *Neurospora* (Sargent et al. 1966), *Drosophila* and *Chlamydomonas* (for review see Feldman 1982).

A strong indication that an endogenous rhythm is involved in *cab* gene expression was demonstrated in tomato plants which were grown without environmental cues. In continuous darkness the

steady-state mRNA level of the Chl a/b-binding proteins continues to oscillate for 4 d, accompanied by a gradual damping of the amplitude (Piechulla 1988). In addition, when plants were exposed to altered light/dark regimes, and during the adaptation to new environments, the typical oscillation pattern remained present (Piechulla 1989). However, the time points of maximum and minimum amplitudes, and the maximum expression level were altered under the modified light situation. Light is known as one of the most effective external factors in resetting and modulating the frequency of the oscillation (Roenneberg and Hastings 1988). To get a better understanding of this circadian rhythm it was necessary to characterize the diurnal fluctuations of the *cab* transcripts in tomato leaves in more detail.

In this article the following questions were addressed to define the oscillations in the levels of the mRNA for Chl a/b-binding proteins in tomato leaves: i) Is the diurnal oscillation affected by the development stage of the leaves? ii) What influence do the circadian phase and the duration of light and darkness have on the level of *cab* expression? iii) Is this typical oscillation pattern of the *cab* mRNA expressed in other monocotyledonous and dicotyledonous plants, and do other genes exhibit a similar diurnal fluctuation pattern?

Material and methods

Plant materials and tissue preparation. Plants were grown in the greenhouse at the university of Göttingen with supplementary light (6 a.m.–10 p.m.) when harvested in January and February 1987 and 1988), and without additional light when harvested in May and June 1988. The temperature was between 20–25° C. The following plants were grown: Tomato (*Lycopersicon esculentum* (Mill.) cv. VFNT LA 1221, cherry line), maize (*Zea mays* L., Mutin, hybrid), soybean (*Glycine max.* L. (Merr.), Efi), french bean (*Phaseolus vulgaris* L., Sachs), mung bean (*Phaseolus aureus* L.), scarlet runner bean (*Phaseolus coccineus* L., Preisgewinner), pea (*Pisum sativum* L., Kleine Rheinländerin), mustard (*Sinapis alba* L., white), barley (*Hordeum vulgare* L., Roland), and wheat (*Triticum aestivum* L. Sperber). To measure diurnal mRNA levels, leaves were harvested from two-week-old plants at 6 a.m., noon, 6 p.m. and midnight (25 January 1988; sunrise 8:11 a.m. and sunset 4:58 p.m.; 13 June 1988; sunrise 4:06 a.m. and sunset 8:37 p.m.) and leaves of five-week-old non-flowering tomato plants at 5 a.m., 9 a.m., noon, 8 p.m. and midnight (2 June 1987; sunrise 5:11 a.m., sunset 9:30 p.m.). In the case of monocotyledonous plants the part from the middle to the tip of a leaf was harvested. To measure mRNA levels during leaf development, the lengths of the terminal leaflets of tomato plants were measured and recorded. The age of the leaves was determined as described in *Results*. Leaves of defined developmental stages of different non-flowering tomato plants (approximately seven weeks old) were harvested in May 1988.

To investigate the influence of the duration of light or darkness, tomato plants were grown hydroponically for four weeks in the greenhouse under the natural day/night cycle (May 1988). Plants were then transferred to the growth chamber (14 h white light (76 $W \cdot m^{-2}$, 30 neon bulbs, Philips TLM, 115 W, 33 RS, double flux)/10 h dark; 70–80% humidity; 20–25° C) for 5 d to adjust to the new light/dark regime. After the appropriate pulse of illumination or darkness, two individual tomato plants were transferred into another growth chamber with or without light, respectively. Leaves were harvested at the indicated time points (light test: transfer time, 12:30 p.m., 7:30 p.m.; dark test: transfer time, 6:30 a.m., 12:30 p.m.; diurnal light-sensitivity test: 6:30 a.m., 9:00 a.m.; 11:00 a.m., 1.00 p.m., 5:00 p.m., 8:00 p.m., 0:00 a.m., 4:30 a.m., and the respective times 4 h later (10:30 a.m., 1:00 p.m., 3:00 p.m., 5:00 p.m., 9:15 p.m., 0:00 a.m., 4:30 a.m., 8:30 a.m.).

Leaflets and leaves were immediately frozen in liquid nitrogen and stored at −50 or −80° C.

Isolation and analysis of RNA. RNA was isolated from 0.25–1.0 g leaf tissue according to the method described by Piechulla et al. (1986). The RNAs from different preparations were standardized and analyzed by spectrophotometric quantitation, by ethidium-bromide fluorescence of the cytoplasmic ribosomal (r)RNA in stained formaldehyde agarose gels and by relative levels of hybridization with heterologous cytoplasmic rDNA. Total RNA samples were spotted onto nylon filter (Hybond N; Amersham Buchler, Braunschweig, FRG) using the dot-blot or slot-blot apparatus. Specific gene probes labeled by nick translation or random priming were used for hybridizations (Table 1). To determine transcript levels of specific mRNAs, dots from hybridized dot blots were cut out and counted in a scintillation counter (Cerenkov counting). Relative mRNA levels were based on two to four hybridizations.

Chlorophyll determination. Leaf tissue was ground in liquid nitrogen and chlorophyll was extracted in 80% acetone (Arnon 1949). The Chl a and b contents were calculated: Chl a = $(E_{663\ nm} \times 12.7) - (E_{645\ nm} \times 2.69)$; Chl b = $(E_{645\ nm} \times 22.9) - (E_{663\ nm} \times 4.68)$; Chl total = $(E_{645\ nm} \times 20.2) + (E_{663\ nm} \times 8.02)$.

Results

The expression of the *cab* genes is at least partially controlled by a circadian rhythm. Several aspects which influence *cab* mRNA accumulation and the amplitude of the transcript level during the circadian oscillation are investigated and presented in this paper. For this purpose, different plant species were grown in the greenhouse or in a growth chamber and leaves were harvested at the indicated time points during the day and night. Total RNA was extracted, applied to dot or slot blots, and hybridized with specific gene probes (Table 1).

Comparison of cab expression at different developmental stages of tomato leaves. To define the developmental stage of tomato leaflets, the lengths of the terminal leaflets were measured and recorded over a period of approximately four weeks. A typical S-shaped growth curve was observed. The ages of the leaves were determined according to the growth curve and based on the plastochron-index

Table 1. Plastic and nuclear gene probes used for hybridization

Gene	Plasmid	Fragment	Size	Plant	Reference
Nuclear					
*Cab*1B	pIA27	HincII-PvuII	0.55 kbp	Tomato	Pichersky et al. 1985
*rbc*S3A	P3–67	XbaI-EcoRI	0.35 kbp	Tomato	Pichersky et al. 1986
Phosphoenolpyruvate carboxylase	pH1	RsaI	0.29 kbp	Maize	Hudspeth et al. 1986
Pyruvate orthophosphate dikinase	pH2	NcoI-PstI	0.2 kbp	Maize	Hudspeth et al. 1986
Triosephosphate isomerase	pMRT1	BamHI-EcoRI	1.15 kbp	Maize	Marchionni and Gilbert 1986
Actin	pMac1	EcoRI-PstI	0.7 kbp	Maize	Shah et al. 1983
α-Amylase	p4868	PstI	1.0 kbp	Wheat	Lazarus et al. 1985
Ubiquitin	pKG3730	PstI	0.2 kbp	Barley	Gausing and Barhardottir 1986
cab	pHvLF2	BglI-EcoRI	1.5 kbp	Barley	K. Apel[1]
*atp*A(mt)	pTA22	EcoRI-BamHI	0.7 kbp	Maize	Braun and Levings 1985
rDNA	pHA2	–	8.7 kbp	Pea	Jörgensen et al. 1981
Chloroplast					
*psb*A	pTB8	XbaI	1.55 kbp	Tobacco	Sugita et al. 1984
*rbc*L	pTB1	BamHI	1.2 kbp	Tobacco	Shinozaki and Sugiura 1982
*psa*A	pSoc1080	PstI	8.2 kbp	Spinach	E. Orozco[2]

[1] Botanical Institute, University of Kiel, FRG
[2] USDA-ARS, University of Illinois, Urbana, USA

model (Erickson and Michelini 1957; Coleman and Greyson 1976). The reference length of 25 mm of the terminal leaflet was arbitrarily set as day zero. A representative example is depicted in Fig. 1A. Logarithmic growth was monitored over a period of approximately one week between leaf ages −4 and +4 d. The stationary phase was defined between days 6 and 20, and senescence, accompanied by the loss of chlorophyll, was detected in leaves older than 10 d. Despite the dramatic chlorophyll degradation, the chlorophyll a/b ratio decreased only slightly from 3.3 in young leaflets to 2.9 in old leaflets (Fig. 1B). Interestingly, during the initial part of the stationary phase, when no significant alterations in the chlorophyll content were observed, the mRNA level of the Chl a/b-binding proteins declined rapidly. Maximum transcript accumulation was monitored at the end of the logarithmic growth of the leaflets (Fig. 1C).

To monitor diurnal mRNA fluctuations, terminal leaflets of defined developmental stages (logarithmic phase = 0.7 ± 1.4 d; 11.3 ± 1.0 d; 15.2 ± 0.8 d; 19.8 ± 0.7 d; 25.6 ± 1.0 d) from different individual plants were harvested at several time points over 60 h (Fig. 2). Relative levels of specific mRNAs were determined by hybridization with the coding region of *cab* 1B gene of the multigene family of tomato. The typical circadian oscillation pattern was observed at different developmental stages of the leaves, indicating that the principle activation/inactivation mechanism remained active throughout most of the lifetime of a leaf. The amplitudes of the oscillations of steady-state mRNA levels, however, varied depending on the developmental stages. The amplitude of the oscillation of *cab* transcript accumulation was highest in young tomato leaves (logarithmic phase). With increasing age a tendency towards amplitude reduction was observed (Fig. 2). In 25 d-old-leaves the amplitude was reduced by approx. 75% compared with logarithmic-phase leaves.

Influence of the duration of light and darkness on the level of cab expression. In tomato plants, grown in a 14 h white light/10 h dark regime, only very low *cab* mRNA levels were detected 10 min before the transition from dark to light. After 6 h of light (12:30 p.m.) the mRNAs had accumulated to high levels, while during further illumination the levels decreased significantly (Fig. 3A). To characterize the accumulation pattern of *cab* mRNAs more precisely, tomato terminal leaflets were harvested and pooled from two individual plants after 0, 1, 2, 4, 6, 8, 10 and 12 h of illumination (Fig. 3B). Highest *cab* steady-state mRNA levels were monitored at 10:30 a.m., 4 h after the transition from dark to light (6:30 a.m.). Further exposure to light did not result in higher mRNA accumulation; indeed, transcript levels decreased, continuously after 10:30 a.m.

In tomato plants which were not exposed to light, the steady-state mRNA level increased 2.5-fold within 6 h, followed by a decrease to an undetectable level at 7:30 p.m. This typical mRNA oscillation during extended dark periods is a strong indication that *cab* gene expression is regulated by

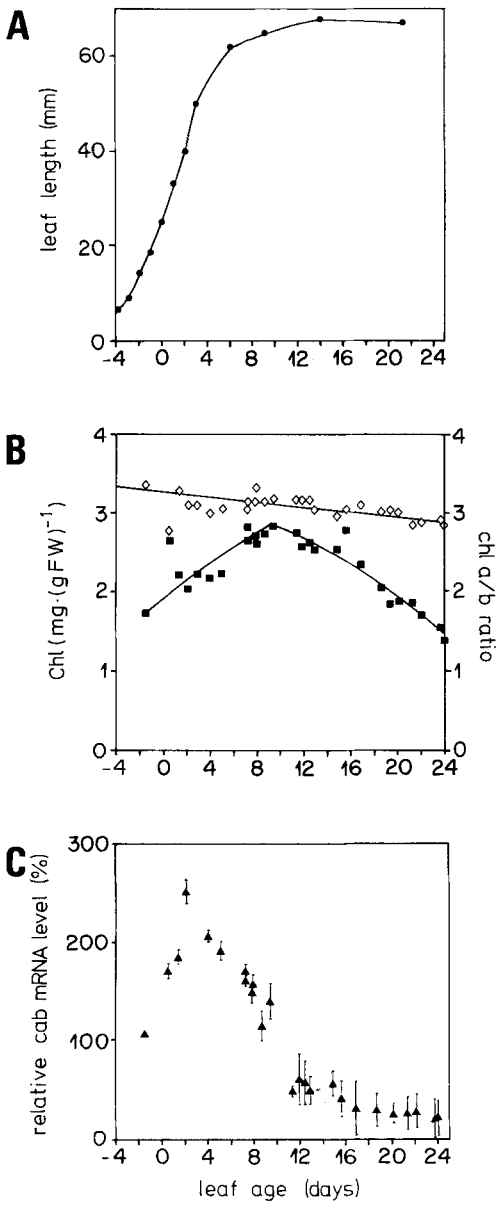


Fig. 1A–C. Alterations of various parameters during tomato leaf development. **A** A representative example of a growth curve of tomato leaves. The length of the terminal leaflet from the leaf of the 11th node was recorded on a seven-week-old tomato plant which was grown in the greenhouse under the natural day/night cycle (June 1988). The length of 25 mm was arbitrarily set as day zero. **B** Determination of the total Chl content (■) and the Chl a/b ratio (◇) during leaf development. Data are based on two measurements of the same leaf collection (seven-week-old tomato plants, harvested 29 March 1988). **C** Determination of relative steady-state mRNA levels of the Chl a/b-binding proteins during tomato leaf development. Thirty probes of different ages were collected between 1 and 2 p.m. (29 March 1988). For each time point, two to eight leaves of the same developmental stage from different individual plants were harvested. A 3-μg sample of total RNA extracts was hybridized with the coding region of the *cab* 1 B gene from tomato. The average of all counts measured at all time points during the development represent 100% transcript level. The data were based on three hybridizations. Errors bars represent ± SE

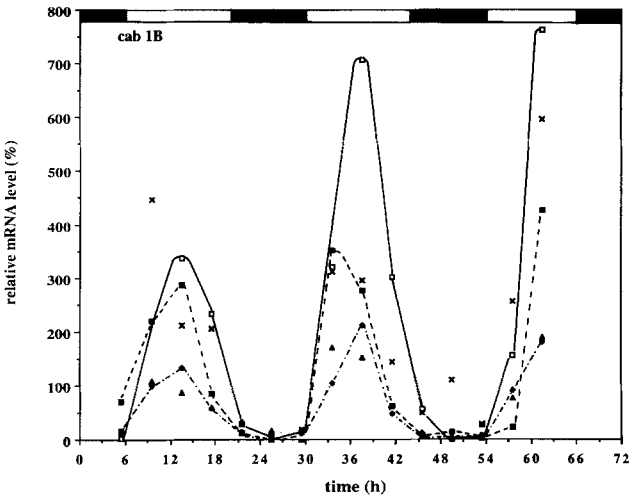


Fig. 2. Diurnal *cab* mRNA oscillations at different stages in the developmental of tomato leaves. Determination of the relative steady-state mRNA levels of the Chl a/b-binding proteins in leaves at the logarithmic growth phase = 0.7 ± 1.4 d (□), and at the ages of 11.3 ± 1 d (×), 15.2 ± 0.8 d (■), 19.8 ± 0.7 d (△), and 25.6 ± 1 d (▲). Six terminal leaflets of the logarithmic phase of two terminal leaflets of the other developmental stages were harvested at 1, 5 and 9 a.m. and p.m. over a period of 60 h. The average of all counts measured at each developmental stage represent 100% relative transcript level. Data are based on two hybridizations. The natural day and night regime is indicated by open and filled bars, respectively

an endogenous rhythm (see also Piechulla 1988). After 1, 2, and 4 h of illumination the *cab* transcripts accumulated gradually at the respective time points, whereas after the transfer to darkness the levels declined with a different rate. In tomato leaves which were not exposed to light the *cab* transcripts at 12:30 p.m. reached a relative level of approx. 40%. In contrast, after 1 h of illumination followed by 5 h of darkness the relative level was reduced to approx. 10%. Similarly, the mRNA level declined after 2 h of light and 4 h darkness, as well as 4 h light and 2 h darkness. This observation is possibly an indication that the stability of *cab* mRNA is altered.

In previous experiments we had already noticed that the duration of darkness was also important for the degree of *cab* mRNA accumulation during the following light phase (Piechulla 1988, 1989). To analyze this observation, two individual tomato plants were transferred after 0, 2, 4, 8, 9 and 10 h of darkness into light, and steady-state mRNA levels were determined at transfer times, at 6:30 a.m. and at 12:30 p.m. In tomato leaves which were kept for 0, 2 and 4 h in the dark only little mRNA accumulation can be detected at 6:30 a.m. and 12:30 p.m. (Fig. 4B). Three- and six-fold elevations in the *cab* transcript levels were monitored

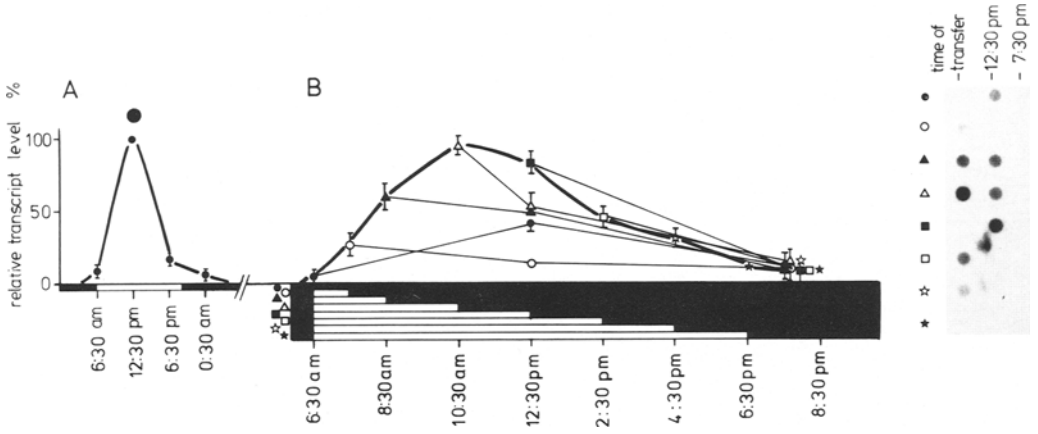


Fig. 3A, B. Influence of the illumination period on the expression of *cab* genes in tomato leaves. Tomato plants were grown for four weeks in the greenhouse under the natural day/night cycle (May 1988) and then transferred to the growth chamber for 5 d (14 h white light/10 h darkness, dark/light transition at 6:30 a.m.). At the end of the dark phase, plants were illuminated for the indicated times and transferred back to darkness. Steady-state mRNA levels of the *cab* proteins were determined by spotting 3 µg of total RNA extract onto dot blots, and specific mRNA was detected by hybridization with random primed *cab* 1B coding region ($7.3 \cdot 10^5$ Bq·µg^{-1}, Cerenkov counting). The autoradiograms were exposed for 23 h at −50° C with an intensifying screen. Dots were cut out and counted. Relative mRNA levels are based on two hybridizations. Error bars represent ± SE. **A** Typical circadian mRNA-oscillation pattern with autoradiogram (*above*) of steady-state *cab* transcript levels determined at the times indicated. **B** Relative steady-state mRNA levels of *cab* transcripts after 0, (●), 1 (○), 2 (▲), 4 (△), 6 (■), 8 (□), 10 (☆), and 12 (★) h of illumination. Transcript levels were determined shortly before the end of the illumination period (= transfer time), at 12:30 p.m. and at 7:30 p.m. (autoradiogram on *left*)

at 12:30 p.m. in tomato plants kept in darkness for 8 or 9 h and 10 h, respectively. These results demonstrate the importance and/or necessity of long dark phases for high levels of *cab* mRNA accumulation during the following illumination phase.

Illumination at different time points during the day. Light is known to influence substantially the expression of photosynthesis-specific genes in seedlings and developed plants (for review see Tobin and Silverthorne 1985; Kuhlemeier et al. 1987). In the previous section it was shown that, in tomato leaves, *cab* mRNA accumulated to maximum levels within 4–5 h of illumination. It was of interest to know what effect a light pulse of 4 h at different time points during the day would have on the pattern of transcript accumulation. To test this, tomato plants were grown in a 14 h white

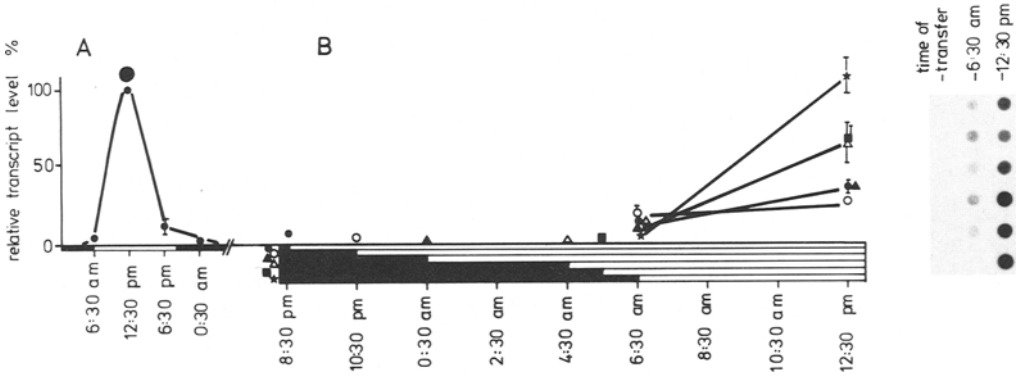


Fig. 4A, B. Influence of the length of the dark period on the expression level of *cab* genes in tomato leaves. Plants were grown and *cab* trancript levels determined as described in the legend to Fig. 3. Relative mRNA levels are based on two hybridizations. Error bars represent ± SE. **A** Typical circadian mRNA-oscillation pattern with autoradiogram (*above*) of steady-state *cab* trancript levels determined at the times indicated. **B** Relative steady-state mRNA levels of *cab* after 0 (●), 2 (○), 4 (▲), 8 (△), 9 (■) and 10 (★) h of darkness. At the end of the light phase (8:30 p.m.) plants were kept in darkness for the indicated times before transferring them back into light. Steady-state mRNA levels of *cab* were determined shortly before illumination (= transfer time), at 6:30 a.m. and at 12:30 p.m. (autoradiogram on *left*)

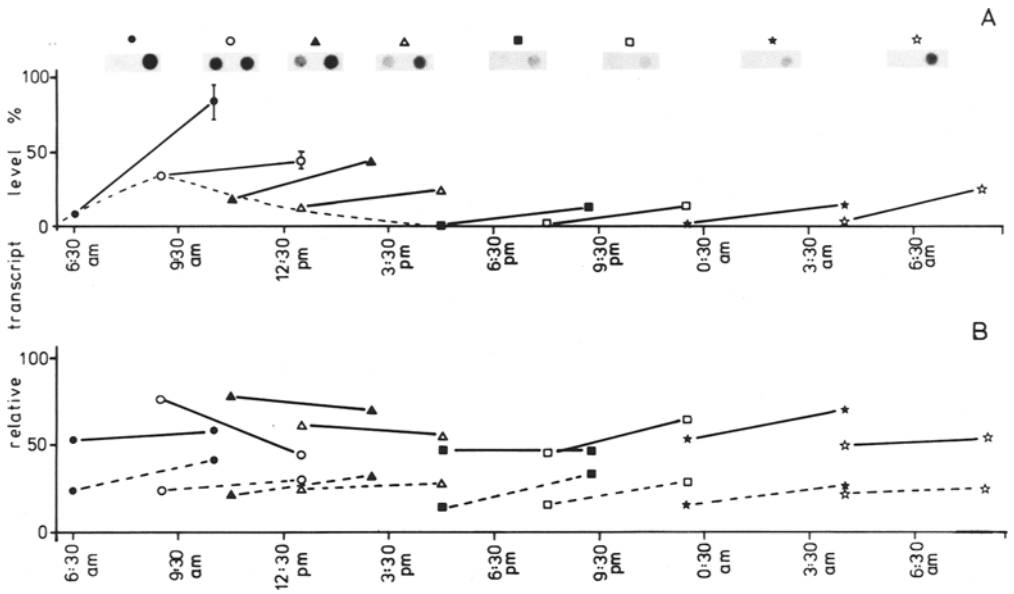


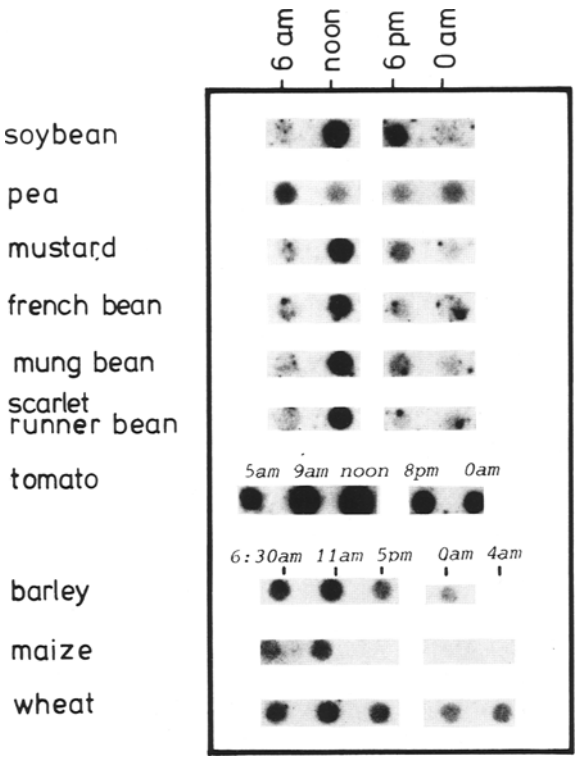
Fig. 5A, B. Influence of the "day time" on the response to illumination. Tomato plants were grown for four weeks in the greenhouse, transferred to the growth chamber for 5 d (14 h white light/10 h darkness, 6:30 a.m. dark/light transition), and kept in complete darkness for 2 d. At the indicated times during the second day of darkness, two tomato plants were illuminated for 4 h. The transfers occurred at 6:30 a.m. (●), 9 a.m. (○), 11 a.m. (▲), 1 p.m. (△), 5 p.m. (■), 8 p.m. (□), 0 a.m. (★) and 4:30 a.m. (☆). Steady-state mRNA levels were determined shortly before illumination (=transfer time) and 4 h later. Three µg of total RNA were spotted onto dot blots and hybridized with "random primed" probes: **A** coding region of *cab* 1 B ($7.3 \cdot 10^5$ Bq·µg^{-1}, Cerenkov counting); **B** (solid line) coding region of *rbc*L ($1.4 \cdot 10^6$ Bq·µg^{-1}, Cerenkov counting), and (interupted line) coding region of *rbc*S 3 A ($1.4 \cdot 10^6$ Bq·µg^{-1}, Cerenkov counting). The autoradiogram in **A** was exposed for 23 h at −50° C with intensifying screens. Dots were cut out and counted. Relative mRNA levels are based on two hybridizations. Error bars represent ± SE

light/10 h dark regime (dark/light transition at 6:30 a.m.), followed by 2 d of complete darkness to reduce the mRNA fluctuations due to the circadian rhythm. During the third day of darkness plants were illuminated for 4 h at various time points during the day. Leaves were harvested from two individual plants at the time of transfer (reflects the expression due to endogenous rhythm) and 4 h later, respectively. The results of this analysis are depicted in Fig. 5A, B. To evaluate the degree of *cab* expression, the steady-state transcript levels after 4 h of light as well as the accumulation rates were compared (Fig. 5A). The light sensitivity during the circadian period was found to vary. High steady-state mRNA levels and accumulation rates (approx. 16% of relative transcript level/hour) were measured only in plants which were illuminated at 6:30 a.m. Minor effects of the light (accumulation rates 2–5% of relative transcript level/hour) are recorded in plants illuminated at the other times during the day.

Additionally, light given at different times during the day was tested for its effect on the mRNA levels of the large (*rbc*L) and small (*rbc*S) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Fig. 5B). Slight increases (1–4.5% of relative transcript level/hour) of small-subunit mRNA were monitored at all time points during the day, and in the case of the large-subunit mRNA few increases and decreases were detected. The differences in light sensitivity during the circadian period demonstrated clearly the dissimilarity of the activation mechanisms that underly the regulation by light induction of photosynthesis-specific nuclear and plastid genes (*rbcS, cab*, and *rbcL*).

Diurnal cab expression in various plant species. For this survey, seven dicotyledonous and three monocotyledonous plants were selected. The plants were grown in the greenhouse, and leaves of soybean, mustard, french bean, scarlet runner bean, mung bean and pea were collected at 6 a.m., noon, 6 p.m. and midnight (January 1988), tomato leaves at 5 a.m., 9 p.m., noon, 8 p.m., and midnight (February 1987), and the leaves of barley, maize and wheat were harvested at 6:30 a.m., 11 a.m., 5 p.m., midnight and 4 a.m. (June 1988).

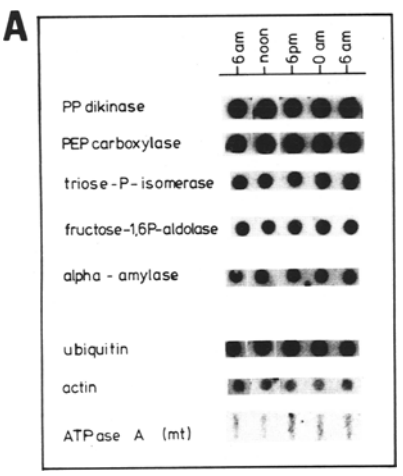
Equal amounts of the total RNA extracts were spotted onto dot blots and hybridized with the Chl a/b-binding protein gene (*cab* 1 B) of tomato. The pea RNA was hybridized with the *cab*-specific probe from barley (Fig. 6). A comparison of the



steady-state transcript levels during a diurnal cycle shows that in all plant species tested – except for pea – substantially elevated *cab* mRNA levels were observed approximately at noon. This result indicates that similar oscillation activation mechanisms are present in monocotyledonous and dicotyledonous plants.

It is interesting to note that increasing steady-state mRNA levels between midnight and 4 or 6 a.m. are detected in pea, barley and wheat. This mRNA accumulation may be due to the annual shift of the time points of sunrise and sunset, since

Fig. 6. Determination of diurnal fluctuations of *cab* mRNA levels in different plant species. RNA was extracted from leaves of dicotyledonous plants at 6 a.m., noon, 6 p.m., and midnight (January 1988), from tomato at 5 a.m., 9 a.m., noon, 8 p.m. and midnight (February 1987), and from barley, maize and wheat at 6:30 a.m., 11 a.m., 5 p.m., midnight and 4 a.m. (June 1988). Three µg of total RNA were spotted onto dot blots and hybridized with nick-translated coding region of *cab* 1B from tomato ($2{,}3 \cdot 10^4$ Bq·µg^{-1}, Cerenkov counting); the pea RNA was hybridized with the coding region of a *cab* gene from barley ($5.2 \cdot 10^4$ Bq·µg^{-1}, Cerenkov counting). The autoradiogram was exposed for 5 d at −50° C with an intensifying screen



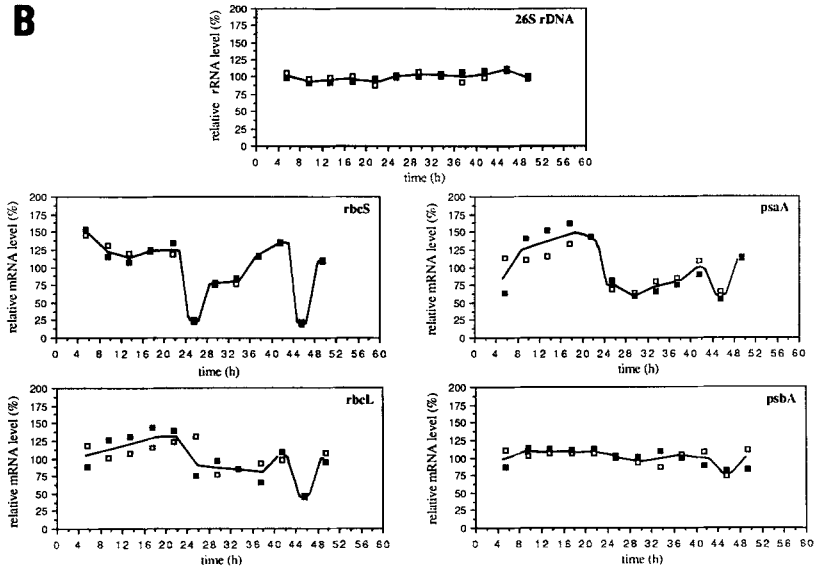


Fig. 7A, B. Diurnal mRNA levels of a variety of genes. **A** Maize leaves. RNA was extracted from maize leaves at 6 a.m., noon, 6 p.m., midnight, and 6 a.m. (June 1988). Three µg of RNA were spotted onto dot blots and hybridized with nick-translated gene probes, specific for enzymes involved in carbon metabolism and other physiological and morphological reactions (Table 1). The autoradiogram was exposed for 4 d (pyruvate orthophosphate (*PP*) dikinase, phosphoenolpyruvate (*PEP*) carboxylase, fructose-1,6-bisphosphate aldolase) and one week (triosephosphate isomerase, α-amylase, ubiquitin, actin, α-subunit of mitochondrial ATPase) at −50° C with an intensifying screen. The specific activities of the probes were: pyruvate orthophosphate dikinase, $2.2 \cdot 10^5$ Bq·µg^{-1}; phosphoenolpyruvate carboxylase, $1.3 \cdot 10^5$ Bq·µg^{-1}; triosephosphate isomerase, $0.83 \cdot 10^4$ Bq·µg^{-1}; fructose-1,6-bisphosphate aldolase, $9.2 \cdot 10^4$ Bq·µg^{-1}; α-amylase, $1.7 \cdot 10^3$ Bq·µg^{-1}; ubiquitin, $1.8 \cdot 10^4$ Bq·µg^{-1}; actin, $4.2 \cdot 10^4$ Bq·µg^{-1} and α-subunit of the mitochondrial ATPase, $1 \cdot 10^4$ Bq·µg^{-1} (Cerenkov counting). **B** Tomato leaves. RNA was extracted from four leaves (17.5 ± 1.2 d old) of two tomato plants (6.5 weeks old, February 1988). Three µg of RNA were spotted onto dot blots and hybridized with nick-translated gene probes (Table 1). The specific activities of the probes were: large subunit of Rubisco, $1.1 \cdot 10^4$ Bq·µg^{-1}; small subunit of Rubisco $3.3 \cdot 10^4$ Bq·µg^{-1}; Q_B-binding protein of photosystem II (PSII), $7.5 \cdot 10^4$ Bq·µg^{-1}; P700 reaction center protein of PSI, $3.8 \cdot 10^4$ Bq·µg^{-1} and rDNA, $7.5 \cdot 10^4$ Bq·µg^{-1} (Cerenkov counting)

maize, barley and wheat plants were harvested in June (sunrise 4:06 a.m., sunset 8:37 p.m.). However, this does not explain the increase of mRNA prior to illumination in pea leaves (sunrise 8:11 a.m., sunset 4:58 p.m.). The phenomenon of elevated transcript levels before pea plants are exposed to light was also documented by Kloppstech (1985).

Analysis of diurnal expression of various genes in maize and tomato leaves. To investigate whether other genes exhibit similar rhythmic expression patterns as monitored for the Chl a/b-binding protein, a number of genes which are involved in carbon metabolism (phosphoenolpyruvate carboxylase, pyruvate orthophosphate dikinase, fructose-1,6-bisphosphate aldolase, α-amylase, triosephosphate isomerase), photosynthetic reactions (large and small subunit of Rubisco, Q_B-binding protein of photosystem II (PSII), P 700 reaction center protein of PSI), and other reactions of physiological and morphological importance (ubiquitin, actin, alpha subunit of mitochondrial ATPase, cytoplasmic ribosomal RNAs) were selected for survey. Maize leaves were harvested at 6 a.m., noon, 6 p.m., midnight and 6 a.m., and tomato leaves at 5 a.m., 9 a.m., noon, 4 p.m., 8 p.m. and midnight. Total RNA extracts from each time point were hybridized with DNA fragments encoding specific gene probes (Table 1). In most cases hybridizations were carried out with homologous probes.

Specific DNA probes coding for enzymes which play a role in carbon metabolism were hybridized to RNA extracts from maize (Fig. 7A). The comparison showed that the steady-state mRNA levels of pyruvate orthophosphate dikinase, phosphoenolpyruvate carboxylase, fructose-1,6-bisphosphate aldolase, alpha amylase and triosephosphate isomerase remain constant throughout the day. No significant periodic fluctuations were observed. Similar results were obtained for the alpha subunit of the mitochondrial ATPase, ubiquitin and actin. The analysis of the expression of photosynthesis-specific genes in tomato leaves exhibits no obvious oscillation pattern (Fig. 7B). Based on the data obtained from this examination, it is very unlikely that any of the genes analyzed here are controlled by a circadian rhythm.

Discussion

The circadian oscillations of the levels of Chl a/b-binding protein mRNA are by now well documented in tomato fruits and leaves (Piechulla and Gruissem 1987; Piechulla 1988, 1989). The present detailed analysis has demonstrated that this mRNA fluctuation pattern (high levels around noon and low levels in the afternoon an during the night) is also present in a number of monocotyledonous and dicotyledonous plant species (Fig. 6). Cycling variations of *cab* mRNA levels were also monitored in petunia (Stayton et al. 1989), in maize (Taylor 1989), in tomato (Giuliano et al. 1988), in wheat (Nagy et al. 1988) and in pea (Kloppstech 1985; Spiller et al. 1987). In addition, it has been demonstrated that expression of the wheat *cab* 1 gene is under the control of a circadian rhythm in transgenic tobacco plants (Nagy et al. 1988). Furthermore, eight *cab* genes of the tomato multigene family exhibit a similar mRNA accumulation pattern (Piechulla and Pichersky, unpublished result). These results together support the notion that this endogenous rhythm affecting *cab* gene expression is highly conserved in higher plants. However, it should be noted that this activation-inactivation mechanism is probably not exclusively restricted to higher-plant species, since *cab* gene expression in synchronized *Chlamydomonas* cells follows a periodic pattern (Shepherd et al. 1983).

Endogenous rhythms with periods of 24 h are common to all eukaryotic organisms. It is speculated that such oscillations arose during evolution because in earlier times organisms performing DNA replication and cell division during the night avoided the mutagenetic effect of high ultraviolet irradiation present during the day (Pittendrigh 1966). Based on this hypothesis, it may be possible to identify other genes which are under the control of a periodic mode. We were particularly interested in genes which code for proteins or enzymes which are involved in photosynthetic reactions and carbon metabolism, since both processes are light dependent. Key enzymes of the Calvin cycle, starch and sugar transformation are activated by light (Stitt et al. 1986; Servaites et al. 1984). The active or inactive status of the enzymes depends on the diurnal cycle. Diurnal changes in enzyme activities can be the result of alterations at the transcriptional, post-transcriptional, translational or post-translational level. The survey presented in this paper (Fig. 7) shows that the transcript levels of none of the 13 genes coding for different cellular functions follow a significant oscillation pattern. Another example of a diurnal fluctuation of mRNA levels was demonstrated by Galangau et al. (1988) for the nitrate-reductase genes from tomato and tobacco. However, the survey presented in this paper demonstrates a typical circadian oscillation pattern for the *cab* transcript level. The

mRNA levels of other photosynthesis-specific genes fluctuate, but not periodically. This leads to the question of what is special about the *cab* genes or their gene products that such a remarkable mechanisms needs to be present to facilitate the expression of LHCPs in many plant species. A possible scenario is based on the function of the LHCPs to bind chlorophyll. Since the synthesis of chlorophyll is strictly dependent on the presence of light (Griffiths et al. 1985), it is likely that the synthesis of the *cab* transcripts and LHCPs is closely coordinated and correlated with the accumulation of chlorophyll.

Despite the activation based on a biological clock, *cab* gene expression is influenced by other circumstances, for example the developmental stage of the leaf (Figs. 1, 2), light, and organ and tissue specificity. It has been known for some time that *cab* genes and other photosynthesis-specific genes are induced by white light in etiolated seedlings and mature plants (Tobin and Silverthorne 1985; Kuhlemeier et al. 1987). It is also generally accepted that the photoreceptor phytochrome is involved in mediating the light response (Pratt 1982). In addition, a detailed analysis in this paper demonstrated the differential accumulation of *cab* transcripts during a period of 24 h (Fig. 5A). Significant *cab*-mRNA accumulation was monitored in plants which were exposed to a pulse of light approximately at the time point of the night/day transition of the previous day, while only a slight increase in the steady-state mRNA levels was observed throughout the rest of the day. The difference of light sensitivity during a circadian period is an important property of the endogenous rhythm in photoperiodism (Mohr and Schopfer 1978, pp. 402–416). The programming or priming of the plants by the biological clock (day/night cycles, time points of dark/light transitions) is probably also responsible for the reduction in the rate of mRNA accumulation after 4–6 h of illumination (Fig. 3; Piechulla 1988, 1989). Similarly, the opening and closing of the flowers of *Kalanchoe blossfeldiana* follows a circadian rhythm, while closure starts 6 h after irradiation.

The results obtained for *cab* expression in tomato leaves favour the hypothesis that, in the hierarchy of the activation mechanisms, the control by an endogenous rhythm ranks higher than that by light induction. The appearance of circadian mRNA-level oscillations may be the consequence of alterations during the process of transcription. It is possible that i) a circadian factor (negative regulator) prevents transcription of the DNA after 4 h of illumination or at time points during the day which are not primed by previous diurnal day/night or light/dark cycles or ii) a circadian factor (positive regulator) has to be synthesized or recovered in order to accumulate and support transcription. The experiments performed so far (Figs. 3, 4, 5) as well as other published results (Piechulla 1989; Nagy et al. 1988) do not favour one or the other possibility.

In contrast to the well-established influence of light on the degree of mRNA accumulation of plastid and nuclear photosynthesis-specific genes in seedlings and developed plants (Kuhlemeier et al. 1987), the effect of the dark period on the level of gene expression has not been investigated in great detail. However, the importance of the dark phase is well documented for the flowering capacity of long-day and short-day plants (Mohr and Schopfer 1978, pp. 312–396). In this paper we showed that the duration of darkness greatly contributes to the amplitude of the oscillation in the expression of the Chl a/b-binding proteins (Fig. 4). Only after a long phase of darkness (approx. 10 h) maximum *cab* mRNA accumulation was observed. The important influence of the dark phase on the steady-state transcript level of *cab* had already been noted in other experiments, for example when the dark phase was reduced from 13 h to 7 h (Piechulla 1989) or when there was a 12-h dark phase present after an extended period of illumination (Piechulla 1988). A balanced duration of light and darkness is probably necessary to optimize and coordinate the synthesis of chlorophyll (Griffith et al. 1985), the expression of the Chl a/b-binding proteins and the assembly of the thylakoid membranes immediately after sunrise.

The highest amplitudes in the oscillations of *cab* mRNA expression were observed in young leaves (Fig. 2). During this developmental stage of the leaves a high turnover rate of the *cab* transcripts is necessary to facilitate this phenomenon. The extent of mRNA synthesis and accumulation is apparently adapted to the needs of the leaves (developmental stage, light intensity, light quality). For other photosynthesis-specific nuclear and plastid genes, e.g. the large and small subunits of Rubisco and Q_B-binding protein, no periodic mRNA fluctuations can be monitored. A regulation mechanism similar to the one controlling the diurnal expression of *cab* genes does not seem to be involved in their expression. The transcript variations are probably the consequence of other effects, e.g. in the case of the small subunit of Rubisco, a change of light intensity caused by shading reduces the mRNA levels rapidly (Prioul and Reyss 1988). Apart from the fact that after the illumina-

tion of etiolated seedlings similar expression patterns are observed for plastid and nuclear photosynthesis-specific genes, the results collected from examinations of leaves and fruits of developed tomato plants support the notion that the two photosynthesis-specific nuclear genes (multigene families), *cab* and *rbcS*, are regulated by different mechanisms.

In this paper we reported on the effect of the developmental stage of the leaves, and the duration of light and darkness, as well as the differential light sensitivity during a circadian period, on the extent of accumulation of *cab* mRNAs in tomato leaves. It remains to be elucidated which *cis*- or *trans*-acting factors or signal chains play a role in controlling these events. In particular, the elements involved in *cab* expression regulated by the biological clock are of interest.

The authors thank Mrs. S. Lechte, Mr. K.H. Lange and Mr. W. Hansmann for taking care of so many tomato plants, Mrs. S. Horticolon for preparing the prints and Dr. H. Bahl for critically reading the manuscript (University of Göttingen, FRG). We thank Dr. K. Apel (University of Kiel, FRG) for the clone pHvLF2, M. Marchionni and W. Gilbert (Harvard University, Cambridge, Mass., USA) for the triosephosphate isomerase clone, J.W. Grula (Phytogen, Pasadena, Calif., USA) for the phosphoenolpyruvate carboxylase and pyruvate orthophosphate dikinase clones, R.B. Meagher (University of Georgia, Athens, USA) for the actin clone and K. Gausing (University of Aarhus, Denmark) for the ubiquitin clone. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to B.P.

References

Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24**, 1–15

Braun, C.J., Levings, C.S. III. (1985) Nucleotide sequence of the F_1-ATPase a-subunit gene from maize mitochondria. Plant Physiol. **79**, 571–577

Bünning, E. (1956) Endogenous rhythms in plants. Annu. Rev. Plant Physiol. **7**, 71–90

Bünsow, R. (1953) Endogene Tagesrhythmik and Photoperiodismus bei *Kalanchoe blossfeldiana*. Planta **42**, 220–252

Coleman, W.K., Greyson, R.I. (1976) The growth and development of the leaf in tomato (*Lycopersicon esculentum*). I. The plastochron index, a suitable basis for description. Can. J. Bot. **54**, 2421–2428

Erickson, R.O., Michelini, F.J. (1957) The plastochron index. Am. J. Bot. **44**, 297–304

Feldman, J.F. (1982) Genetic approaches to circadian clocks. Annu. Rev. Plant Physiol. **33**, 583–608

Galangau, F., Daniel-Vedele, F., Moureaux, T., Dorbe, M.-F., Leydecker, M.-T., Caboche, M. (1988) Expression of leaf nitrate reductase genes from tomato and tobacco in relation to light-dark regimes and nitrate supply. Plant Physiol. **88**, 383–388

Gausing, K., Barkardottir, R. (1986) Structure and expression of ubiquitin genes in higher plants. Eur. J. Biochem. **158**, 57–62

Giuliano, G., Hoffmann, N.E., Ko., K., Scolnik, P.A., Cashmore, A.R. (1988) A light-entrained circadian clock controls trancription of several plant genes. EMBO J. **7**, 3635–3642

Griffiths, W.T., Kay, S.A., Oliver, R.P. (1985) The presence of photoregulation of protochlorophyllide reductase in green tissue. Plant Mol. Biol. **4**, 13–22

Hudspeth, R.L., Glackin, C.A., Bonner, J., Grula, J.W. (1986) Genomic and cDNA clones for maize phosphoenolpyruvate carboxylase and pyruvate, orthophosphate dikinase: Expression of different gene-family members in leaves and roots. Proc. Natl. Acad. Sci. USA **83**, 2884–2888

Jerebzoff, S. (1986) Cellular circadian rhythms in plants: recent approaches to their molecular bases. Physiol. Veg. **24**, 367–376

Jörgensen, R.A., Cuellar, R.E., Thompson, W.F. (1981–1982) Modes and tempos in the evolution of nuclear encoded ribosomal genes in legumes. Carnegie Inst. Washington Yearb. **81**, 98–101

Kloppstech, K. (1985) Diurnal and circadian rhythmicity in the expression of light-induced plant nuclear messenger RNAs. Planta **165**, 502–506

Kuhlemeier, C., Green, P.J., Chua, N.H. (1987) Regulation of gene expression in higher plants. Annu. Rev. Plant. Physiol. **38**, 221–255

Lazarus, C.M., Baulcombe, D.C., Martienssen, R.A. (1985) Alpha amylase genes of wheat are two multigene families which are differentially expressed. Plant Mol. Biol. **5**, 13–24

Marchionni, M., Gilbert, W. (1986) The triosephosphate isomerase gene from maize: introns antedate the plant-animal divergence. Cell **46**, 133–141

Mohr, H., Schopfer P. (1978) Lehrbuch der Pflanzenphysiologie, 2nd edn. Springer, Berlin Heidelberg New York

Nagy, F., Kay, S.A., Chua, N.H. (1988) A circadian clock regulates transcription of the wheat cab-1 gene. Genes Dev. **2**, 376–382

Pichersky, E., Bernatzky, R., Tanksley, S.A., Breidenbach, R.W., Kausch, A.P., Cashmore, A.R. (1985) Molecular characterization and genetic mapping of two clusters of genes encoding chlorophyll a/b-binding proteins in *Lycopersicon esculentum* (tomato). Gene **40**, 247–258

Pichersky, E., Bernatzky, R. Tanksley, S.D., Cashmore, A.R. (1986) Evidence for selection as a mechanism in the concerted evolution of *Lycopersicon esculentum* (tomato) genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Proc. Natl. Acad. Sci. USA **83**, 3880–3884

Piechulla, B. (1988) Plastid and nuclear mRNA fluctuations in tomato leaves – diurnal and circadian rhythms during extended dark and light periods. Plant Mol. Biol **11**, 345–353

Piechulla, B. (1989) Changes of the diurnal and circadian (endogenous) mRNA oscillations of the chlorophyll a/b-binding protein in tomato leaves during altered day/night (light/dark) regimes. Plant Mol. Biol. **12**, 317–327

Piechulla, B., Gruissem, W. (1987) Diurnal mRNA fluctuations of nuclear and plastid genes in developing tomato fruits. EMBO J. **6**, 3593–3599

Piechulla, B., Pichersky, E., Cashmore, A.R., Gruissem, W. (1986) Expression of nuclear and plastid genes for photosynthesis-specific proteins during tomato fruit development and ripening. Plant Mol. Biol. **7**, 367–376

Pittendrigh, S.C. (1966) The circadian oscillation in *Drosophila pseudoobscura* pupae: a model for the photoperiodic clock. Z. Pflanzenphysiol. **54**, 275–307

Pratt, L.H. (1982) Phytochrome: the protein moiety. Annu. Rev. Plant Physiol. **33**, 557–582

Prioul, J.L., Reyss, A (1988) Rapid variations in the content

of the RNA of the small subunit of ribulose-1,5-bisphosphate carboxylase of mature tobacco leaves in response to localised changes in light quantity. Relationships between the activity and quantity of the enzyme. Planta **174**, 488–494

Roenneberg, T., Hastings, J.W. (1988) Two photoreceptors control the circadian clock of a unicellular alga. Naturwissenschaften **75**, 206–207

Sargent, M.L., Briggs, W.R., Woodward, D.O. (1966) The circadian nature of a rhythm expressed by an invertase-less strain of *Neurospora crassa*. Plant Physiol. **41**, 1343–1349

Satter, R.L., Galston, A.W. (1981) Mechanisms of control of leaf movements. Annu. Rev. Plant Physiol. **32**, 83–110

Schweiger, H.G. (1969) Cell biology of *Acetabularia*. Curr. Top. Microbiol. Immunol. **50**, 1–36

Servaites, J.C., Torisky, R.S., Chao, S.F. (1984) Diurnal changes in ribulose-1,5-bisphosphate carboxylase activity and activation state in leaves of field-grown soybeans. Plant Sci. Lett. **35**, 115–121

Shah, D.M., Hightower, R.C., Meagher, R.B. (1983) Genes encoding actin in higher plants: intron positions are highly conserved but the coding sequences are not. J. Mol. Appl. Genet. **2**, 111–126

Shepherd, H.S., Ledoigt, G., Howell, S.H. (1983) Regulation of the light-harvesting chlorophyll-binding protein (LHCP) mRNA accumulation during the cell cycle in *Chlamydomonas reinhardii*. Cell **32**, 99–107

Shinozaki, K., Sugiura, M. (1982) The nucleotide sequence of the tobacco chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Gene **20**, 91–102

Spiller, S.C., Kaufman, L.S., Thompson, W.F., Briggs, W.R. (1987) Specific mRNA and rRNA levels in greening pea leaves during recovery from ironstress. Plant Physiol. **84**, 409–411

Stayton, M., Brosio, P., Dunsmuir, P. (1989) Photosynthetic genes of *Petunia* (Mitchell) are differentially expressed during the diurnal cycle. Plant Physiol. **89**, 776–782

Stitt, M., Mieskes, G., Söling, H.D., Grosse, H., Heldt, H.W. (1986) Diurnal changes of fructose-6-phosphate,2-kinase and fructose-2,6-bis-phosphatase activities in spinach leaves. Z. Naturforsch. **41c**, 291–296

Sugita, M., Sugiura, M. (1984) Nucleotide sequence and transcription of the gene for the 32000 dalton thylakoid membrane protein from *Nicotiana tabacum*. Mol. Gen. Genet. **195**, 308–313

Taylor, W.C. (1989) Transcriptional regulation by a circadian rhythm. Plant Cell **1**, 259–264

Tobin, E., Silverthorne, J. (1985) Light regulation of gene expression in higher plants. Annu. Rev. Plant Physiol. **36**, 569–593

Vince-Prue, D. (1983) Photomorphogenesis and flowering. In: Encyclopedia of Plant Physiol.; N.S., vol 16B: Photomorphogenesis, pp. 458–490, Shropshire, W., Jr., Mohr, H., eds. Springer, Berlin Heidelberg New York

Received 18 January; accepted 26 June 1989