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Regular paper

Circadian oscillations of *Lhc* mRNAs in a photoautotrophic cell culture of *Lycopersicon peruvianum*

Lars Winter[1], Susanne Stöcker[2], Nicole Merforth[1], Hans-Peter Mühlbach[2] & Birgit Piechulla[1,*]

[1]*Institut für Biochemie der Pflanze, Untere Karspüle 2, 37073 Göttingen, Germany;* [2]*Institut für Allgemeine Botanik, Ohnhorststr. 18, 22609 Hamburg, Germany;* **Author for correspondence*

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Abstract

Fourteen genes encoding proteins of the light harvesting complex (*Lhc*) are expressed in a photoautotrophic cell culture from the wild species of tomato (*Lycopersicon peruvianum*). For two genes, *Lhc*a2 (cab7) and *Lhc*b2*1 (cab4), a rhythmic oscillation of the transcript accumulation is observed under light/dark and constant dark conditions indicating that gene expression is controlled by a circadian clock in the tomato cell culture. The circadian expression of the *Lhc* genes remains present after application of 2,2′-dipyridyl. However, the amplitude of *Lhc* mRNA oscillations and the photosynthetic capacity (Fmax/Fo) decrease significantly. The transcript accumulations of *psb*A, *rbc*S and *rbc*L are less or not at all affected by 2,2′-dipyridyl.

Introduction

Chlorophyll molecules together with proteins form highly organized complexes in membranes that function as light harvesting systems in plants as well as in bacteria. Recently, crystals of the light-harvesting complexes became available revealing that the chlorophyll molecules are attached to the polypeptide by coordination of the central magnesium to polar amino acid side chains or main chain carbonyls in the hydrophobic interior of the complex. Each polypeptide organizes a minimum of twelve chlorophylls and two carotenoids (xanthophylls) (Kühlbrandt et al. 1994). A successful complex formation is dependent on the availability of pigments and proteins. In the chlorophyll *b* deficient barley chlorina mutant few if any stable complexes are assembled (Terao and Katoh 1989; Preiss and Thornber 1995) and the components by themself degrade rapidly; apoproteins are destroyed by proteolysis (Apel and Kloppstech 1978, 1980) and the pigments are destroyed by photooxidation (Prasil et al. 1992). The stability and the exact half life times of the light-harvesting complex proteins (LHC) of higher plants is still unknown. This question became even more challenging when diurnal oscillations of the *Lhc* mRNAs and LHC protein synthesis were observed (Piechulla and Gruissem 1987; Piechulla 1993, Riesselmann and Piechulla 1992). Based on our calculations only less than 1% of the LHC proteins are turned over within one day. Although this represents a very small amount of diurnal protein synthesis, it is striking that both, the transcript and the protein synthesis, are under the control of a circadian clock. Apparently, a sophisticated (since the molecular basis of this mechanism is presently not understood) regulatory machinery is needed to precisely direct the synthesis to a particular time of the day. A further indication for the importance of this control mechanism is the conservation during evolution; this diurnal expression pattern is present in all divisions of the plant kingdom, with the exception of coniferophytina (Oberschmidt et al. 1995).

The stoichiometric relationship of the pigments and apoproteins present in the cell determines the LHC complex formation. Previous studies indicate that a 1.5 and 2.3 fold increase of chlorophyll *a* and *b*, respectively, reveals a 2.4 fold increase of the LHC II pro-

teins of *Hordeum vulgare* (Tanaka et al. 1994). Similar results were obtained in the algae *Scenedesmus obliquus* (Hermsmeier et al. 1994). If, for example chlorophyll cannot be synthesized in appropriate amounts it would be interesting to monitor the effect on the accumulation of the apoproteins. In the present study 2,2′-dipyridyl was used to prevent heme formation (Castelfranco and Beale 1983) and the influence on transcript levels of several photosynthesis related proteins were analysed in the green, photoautotrophic cell culture of *Lycopersicon peruvianum*. Special emphasis was placed on the effect of 2,2′-dipyridyl on the circadian expression of the *Lhc* mRNAs.

Methods

Plant material

The photoautotrophic cell culture of *L. peruvianum* was established from a heterotrophic cell suspension by stepwise reduction of the sucrose content in the culture medium and selection of spontaneously developed green cell colonies (Stöcker et al. 1993). Cells were grown in 50 ml MS medium (Sigma M5519) in two-tire vessels containing a 2 M K_2CO_3/$KHCO_3$ buffer which provided 2% CO_2 in the gas atmosphere (Hüsemann and Barz 1977). The cells were maintained on rotary shakers (120 rpm) at 26 °C/21 °C (day/night) with 16 h of white light/8 h of darkness (light phase from 11 a.m. till 3 a.m.; 195 mE s^{-1} m^{-2}, Osram Lumilux 58W/32). To determine the individual *Lhc* mRNAs, cells of the 50 ml culture were harvested after four hours of illumination. To determine the effect of the inhibitor, 10 ml samples were withdrawn at indicated time points and divided into aliquots (1 ml for determination of the cell number; 1 ml for determination of photosynthetic capacity of PS II; 2.5 ml for RNA extractions; 5.5 ml for determination of the chlorophyll content). Immediately after the first time point was taken from the cell culture, 2,2′-dipyridyl was added to give a final concentration of 1 mM.

Determination of cell number, chlorophyll content and photosynthetic capacity

Cell number. Cells of 50–100 mg fresh weight were resuspended in 10% Chrom-VI-oxid and incubated for 10 min at 60 °C. Cell number was determined in a Fuchs-Rosenthal-Zählkammer.

Chlorophyll content. Extraction was performed according to Ziegler and Egle (1965). Chlorophyll *a* and *b* were calculated based on the equations: chl *a* (mg/ml) (E_{664} × 11.78) - (E_{647} × 2.29) and chl *b* (mg/ml) = (E_{647} × 20.05) - (E_{664} × 4.47).

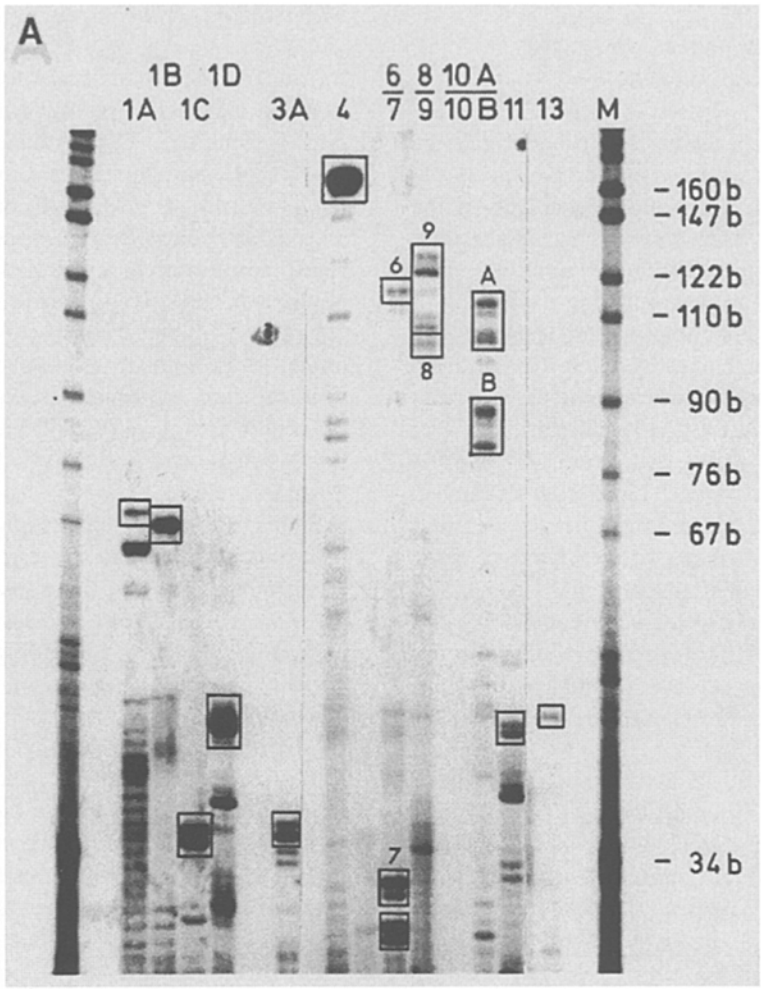
Photosynthetic capacity. The medium of 1 ml cell culture sample was removed. To completely oxidise the photosystems cells were kept in darkness for 30 min. Fo and Fmax fluorescence was determined based on pulse amplified modulated fluorescence measurements (pamf, Schreiber et al. 1986) with a chlorophyll fluorimeter (Walz, Effeltrich, Germany).

RNA extraction, primer extension and dot blot hybridization

Total RNA extractions to determine steady-state mRNA levels of individual *Lhc* genes (Fig. 1) from *L. peruvianum* were performed according to Kellmann et al. (1990, 1993). To determine transcript levels by dot blot hybridizations RNA was extracted according to Piechulla et al. (1986). Three mg of total RNA was spotted onto nylon membranes and hybridized with random primed probes. Specific gene probes were used for the hybridizations: *Lhc*b 2*1 (cab4; Hind III −Bgl I, Pichersky et al. 1987), *Lhc*a 2 (cab7; EcoRI− Pst I, Pichersky et al. 1988), *psb*A, *rbc*L, *rbc*S3A (see Meyer et al. 1989). To determine transcript levels of specific mRNAs, dots of the hybridized dot blots were cut out and counted in a scintillation counter (Cerenkov counting). The average of all counts of one set of experiments was equal to 100%. The relative mRNA levels within one experiment were calculated on the basis of this value.

Nomenclature of the light harvesting complex protein genes (Jansson et al. 1992)

The light harvesting proteins of PS I: *Lhc*a1 (cab6A/B), *Lhc*a2 (cab7), *Lhc*a3 (cab8) and *Lhc*a4 (cab11, 12). The light harvesting proteins of PS II are encoded by *Lhc*b 1 (cab1A-1D, cab3A-3C), *Lhc*b2 (cab4, 5), *Lhc*b3 (cab13, 14, 15, 16), *Lhc*b5 (cab9) and *Lhc*b6 (cab10A/B).



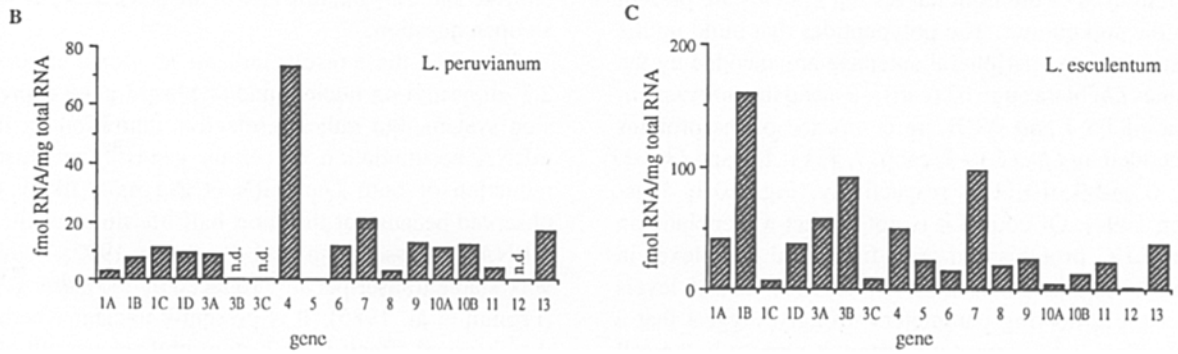


Fig. 1. Steady-state mRNA levels in cell cultures of *L. peruvianum* (A, B) and plants of *L. esculentum* (C). Four hours after the dark to light transition cells were harvested and RNA was extracted. Specific oligonucleotides were used to determine mRNA levels of individual *Lhc* mRNAs in *L. peruvianum*. Primer extended DNA fragments were visualized on autoradiograms (A). Marker sizes are shown on the right. (B) Steady-state levels of 14 *Lhc* mRNAs of *L. peruvianum* are presented in fmol/mg total RNA. (C) Steady-state levels averaged of RNA levels from leaves, green fruit, stems and sepals of 18 *Lhc* mRNAs of *L.esculentum* are presented in fmol/mg total RNA (Kellmann et al. 1993). n.d. not detectable.

Results and discussion

Light-harvesting system in the cells of a photoautotrophic tomato cell culture

Plant cell cultures are a useful tool to investigate the effects of various substances when whole plant experiments are not applicable. Continuous adaptation of the wild tomato species *L. peruvianum* lead to a stable photoautotrophic cell culture (Stöcker et al. 1993). This cell culture was used to determine the expression of individual *Lhc* genes. Four hours after the beginning of illumination cells were harvested, RNA was extracted and individual *Lhc* mRNAs were quantitated by primer extension (Fig. 1A and B). The specific primers were previously successfully used to determine *Lhc* mRNA concentrations in *Lycopersicon esculentum* (Kellmann et al. 1993). The transcripts of fourteen *Lhc* genes were detectable (Fig. 1A). They are differentially expressed and the mRNA levels of almost all genes reach a level between 10–20 fmol/mg RNA, while *Lhc*b2*1 (cab4) mRNA is four-fold higher (Fig. 1B). Most transcript levels in *L. esculentum* (averaged from leaves, stems, sepals and green fruits, Kellmann et al. 1993) (Fig. 1C) are between 5 and 50 fmol/mg RNA, while *Lhc*b1*2, 1*5 and *Lhc*a2 (cab1B, 3B and 7) reach 100 to 160 fmol/mg RNA. The different levels of the individual *Lhc* mRNAs are most likely due to the different growth and environmental conditions existing between cell cultures and whole plants. Despite the fact that the amounts of *Lhc* mRNAs are lower in the cell culture of *L. peruvianum* most (if not all) of the mRNAs which encode the different types of apoproteins of the subcomplexes of the light harvesting systems are present in the cell culture. The polypeptides that build up the trimers of the peripheral antennae are encoded by the genes *Lhc*b1 through b2 (cab1 – 3), and the inner antennae of PS I and PS II are composed of the proteins encoded by *Lhc*a 1 to 4 (cab6, 7, 8, 11, 12) and *Lhc*b3, 5, 6 (cab9, 10, 13), respectively (Fig. 20 in Jansson 1994). Of course it is not correct to conclude on the LHC proteins and their functional complexes in the thylakoid membrane solely based on mRNA levels but the following parameters strongly suggest that a complete light harvesting system is present in the cell culture. Similar values are measured in the cell culture and in leaves of whole plants, (i) the chlorophyll content (Stöcker et al. 1993), (ii) the chl *a*/*b* ratio of 3 (Stöcker 1993), and (iii) the photosynthetic capacity (pamf, 5.17 compared to 5.21 in leaves; Stöcker 1993).

Effect of 2,2′-dipyridyl on the expression of photosynthesis related genes

2,2′-dipyridyl prevents heme formation by chelating iron and magnesium protoporphyrin IX monomethyl ester accumulates (Castelfranco and Beale 1983). 1 mM of this inhibitor in the tomato cell culture does not alter the total chlorophyll content (dipyridyl treated cells and control cells: 3.5 mg/g dry weight). This result indicates no or a very slow turnover of chlorophylls and most likely also of the stable integrated chlorophyll-binding proteins of the thylakoid membranes. More dramatic is the effect on the total photosynthetic capacity, which is reduced to approximately 1/3 within four days (Fig. 2) and on the steady-state levels of two *Lhc* genes (Fig. 3A). The steady-state mRNA levels of *Lhc*b2*1 (cab4) and *Lhc*a2 (cab7) exhibit a 90% reduction within four days, while in control cells the levels decrease only by 10–15%. No effect of dipyridyl on the transcript level was measured for the plastid encoded *psb*A gene (encoding the Q_B-binding protein of PS II) (Fig. 3A).

The transcript levels of photosynthesis related genes encoding the small and large subunit of the ribulose-bisphosphate carboxylase/oxygenase (RuBisCO) are presented in Fig. 3B. After application of 2,2′-dipyridyl the mRNA levels of the *rbc*S genes (nuclear encoded) decline slightly but not significantly more than in the untreated cell culture, while the transcript level of the large subunit (*rbc*L, plastid encoded) exhibit approximately a 50% reduction within four days. Whether the reduced level of *rbc*L transcripts has a consequence on the formation of functional RuBisCO enzyme and CO_2 fixation rate in the cell culture is still an open question.

Together, these results indicate no global effect of 2,2′-dipyridyl on nuclear and/or plastid gene expression system, but rather a selective inhibition on the mRNA accumulation of certain genes. The drastic reduction of both *Lhc* mRNAs can most likely be observed because of the short half life times of these mRNAs (Riesselmann and Piechulla 1992), while very stable transcripts are produced by the *psb*A gene (Fromm et al. 1985). It is presently unclear whether the observed effects by 2,2′-dipyridyl are a result of a specific inhibition of chlorophyll biosynthesis or due to a general inhibition of heme. Furthermore, it is also likely that the accumulation of Mg-protoporphyrin IX monomethyl ester causes alterations at mRNA levels which then lead to an insufficient supply of LHC II protein and further to a degradation of chlorophyll due

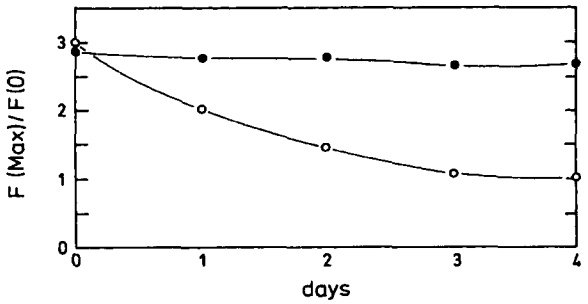


Fig. 2. Determination of photosynthetic capacity (Fmax/Fo) in *L. peruvianum*, control (open circles) and 2,2′-dipyridyl treated (filled circles) cell cultures.

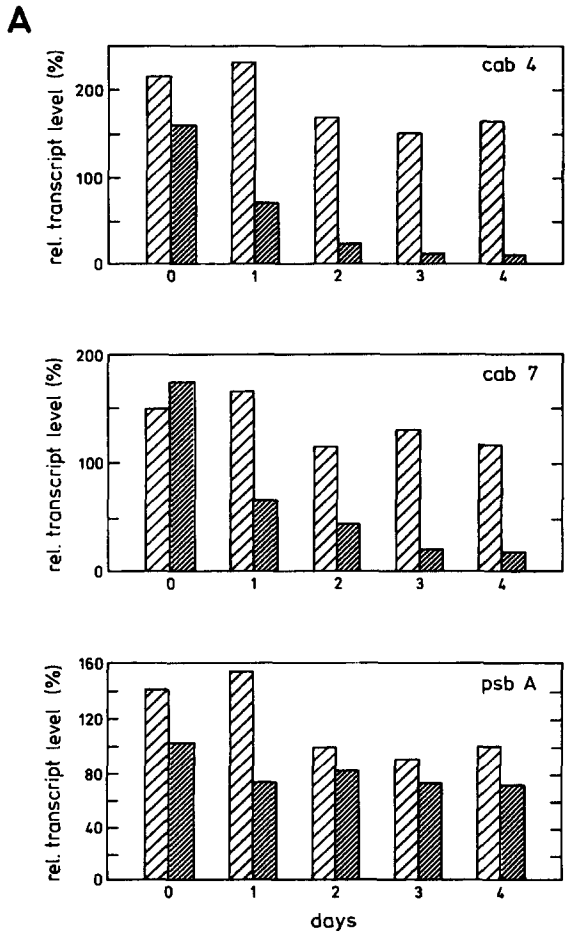


Fig. 3. Determination of relative steady-state mRNA levels of thylakoid membrane proteins (A) and subunits of ribulose-bisphosphate carboxylase/oxygenase (B) in control (left bar) and 2,2′-dipyridyl treated (right bar) cell cultures of *L. peruvianum*. Cells were harvested for five days always at 1 pm. mRNA levels of two light harvesting complex proteins, *Lhcb* 2*1 (cab4), *Lhca* 2 (cab7), and the Q_B-binding protein, *psbA* (A), and the transcript levels of the small (*rbcS*) and large (*rbcL*) subunit of RubisCO (B) were determined. Presented data are the average of three hybridizations.

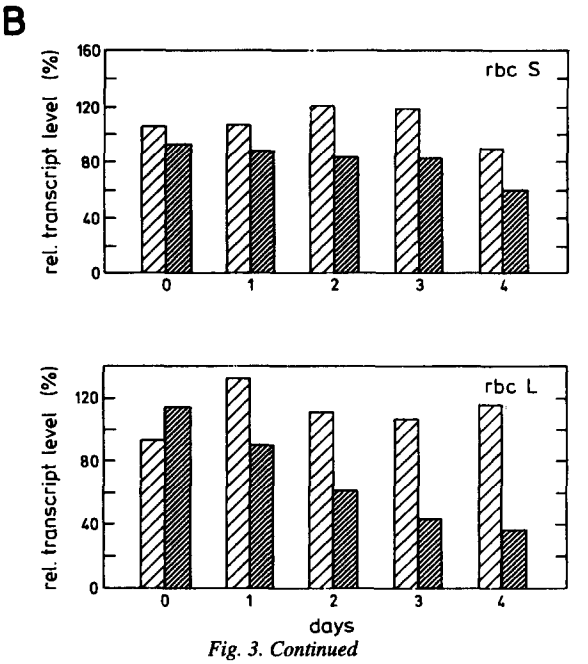


Fig. 3. Continued

to photooxidation. To clarify this subject, inhibitors would be useful which have an exclusive side of action within the chlorophyll synthesis pathway (to our knowledge presently not available) and/or experiments with mutants may help to discriminate between the above mentioned possibilities.

Circadian Lhc *mRNA accumulation after 2,2′-dipyridyl application*

Recently it was demonstrated that the LHC II and chlorophyll synthesis is coordinated (Beator et al. 1992). Furthermore, the chlorophyll synthesis itself occurs with a rhythmic pattern since the 8-aminolevulinic acid synthesis is under circadian control in heat shock synchronized barley etiolated seedlings (Beator and Kloppstech 1993). Based on these results it is very likely that the same circadian clock regulates both, the apoprotein and the pigment formation. Questions remain whether the same or different regulatory pathways exist and/or whether crosstalk between these pathways allow additional coordination. To get some indications to this problem the effect of 2,2′-dipyridyl on the circadian accumulation of *Lhcb2*1* (cab4) and *Lhca2* (cab7) transcripts was registered in the cell culture of *L. peruvianum*. Under light/dark cycles (Fig. 4A and B) mRNA levels reach a maximum at 5 p.m., which is six hours after the dark to

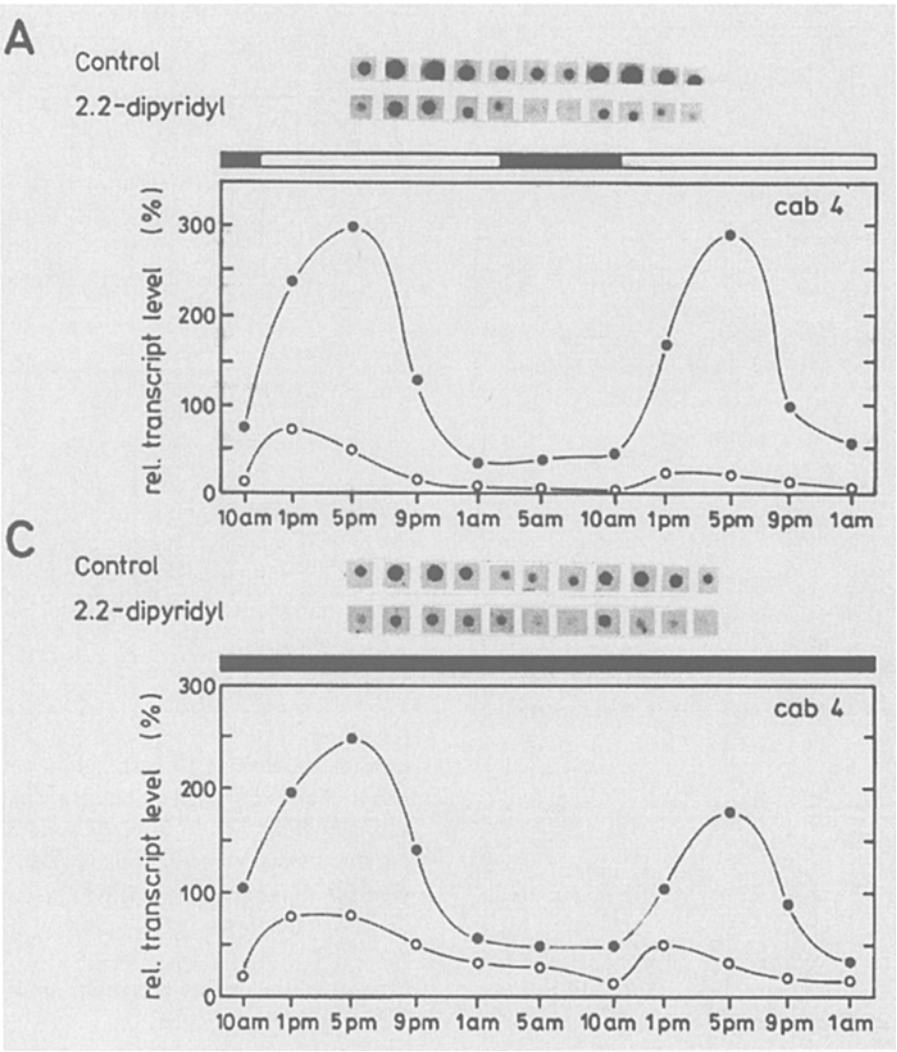


Fig. 4. Circadian mRNA oscillations of light harvesting complex protein mRNAs in control and 2,2′-dipyridyl treated cell cultures of *L. peruvianum*. Steady-state mRNA levels of *Lhc*b2*1 (cab4) (A and C) and *Lhc*a2 (cab7) (B and D) were determined under light/dark conditions (A and B) and constant darkness (C and D) in control (filled circles) and treated (open circles) cell cultures. The results of the dot blot hybridisations are presented in the upper part of each panel.

light transition. Thereafter, the levels decrease significantly (3–6 fold). The transcripts are detectable during the dark phase. Two days in constant darkness (Fig. 4C and D) exhibit a light independent mRNA accumulation, with decreasing amplitudes. The application of 2,2′-dipyridyl reduces *Lhc* mRNA amplitudes (similarly as Fig. 3A) under light/dark and in continuous darkness, but the circadian pattern remains present. Apparently, 2,2′-dipyridyl has no inhibitory effect on the components that play a role in mediating the circadian clock.

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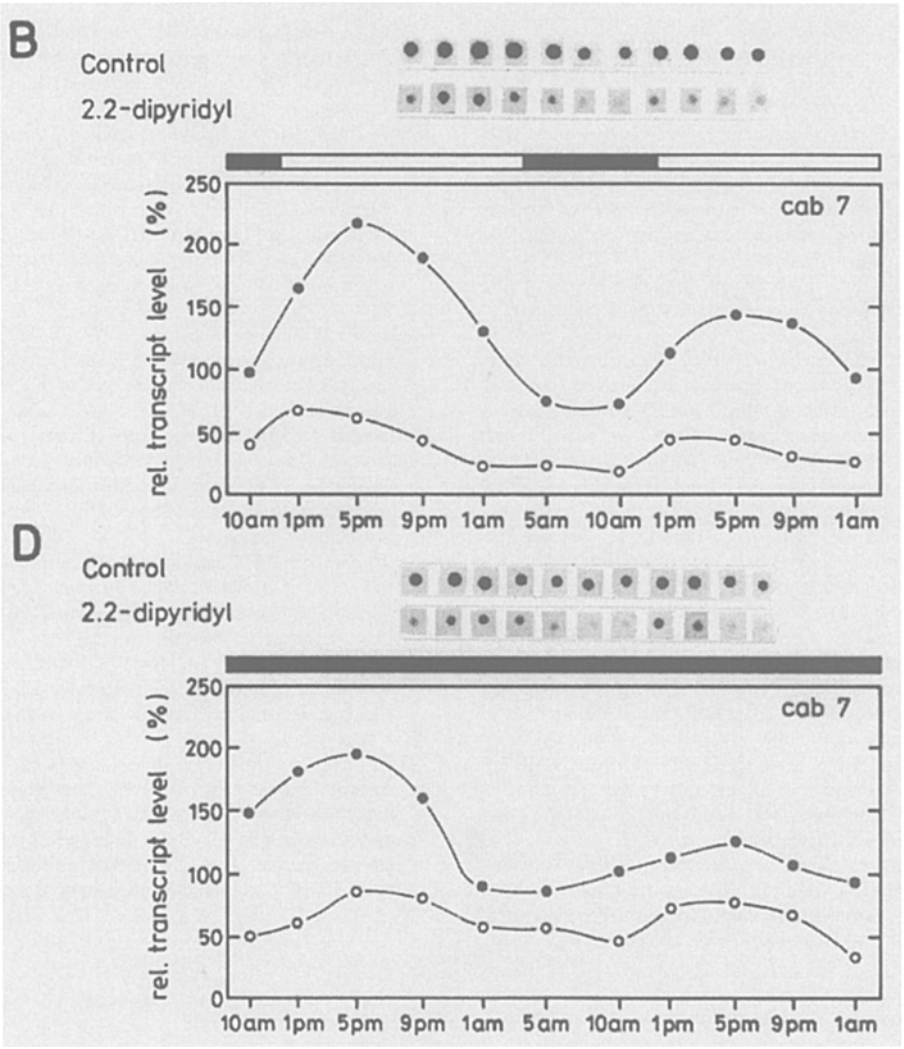

Fig. 4. Continued

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