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The Circadian Oscillator Coordinates the Synthesis of Apoproteins and Their Pigments during Chloroplast Development[1]

Jens Beator and Klaus Kloppstech*

Institut für Botanik, Universität Hannover, Herrenhäuser Strasse 2, 3000 Hannover 21, Germany

Greening has been studied at circadian times of maximal and minimal levels of mRNA for the light-harvesting chlorophyll *a/b* binding protein in photosystem II (Cab mRNA) after circadian synchronization of etiolated barley plantlets (*Hordeum vulgare* cv Apex) by heat-shock treatments. It was found that greening occurs faster and without a lag period when illumination was started at the time of maximal Cab mRNA accumulation. This holds true for the rate of accumulation of Cab and early light-inducible protein mRNAs, the levels of their correspondent proteins, and the levels of chlorophyll *a* and *b*. When illumination was started at the time of Cab mRNA minimum, a lag in the appearance of all components mentioned above was observed. Under these conditions, the lag in chlorophyll *b* accumulation was by far more pronounced than that found for chlorophyll *a*. The circadian oscillation in the capacity of chlorophyll synthesis appears to be controlled via δ-aminolevulinic acid (δ-ALA) synthesis. δ-ALA accumulation after levulinic acid treatment is itself under circadian control; the maxima in stationary concentrations coincide with those of Cab mRNA levels. The amounts of protochlorophyllide and photoconvertible protochlorophyllide showed only minor differences between circadian minima and maxima, the levels being slightly lower during the time of minimum.

One of the model systems with which to investigate chloroplast development is the monocotyledonous leaf (Baker and Leech, 1977). Within a few hours after transfer into light of moderate intensity, the etioplast develops into a functional chloroplast. This process is primarily under transcriptional control as far as gene expression in the nucleo-cytosolic compartments is concerned (Gallagher and Ellis, 1982). However, regulation at other levels has been observed. Translational control plays an eminent role during the expression of plastid DNA-encoded proteins (Mullet, 1988). In both systems, posttranslational regulation has been detected, especially during the assembly of pigment protein complexes; this complex formation is mutually dependent on both partners, because free pigments are destroyed by photooxidation (Prasil et al., 1992) and apoproteins are presumably destroyed by proteolysis (Apel and Kloppstech, 1978, 1980), the mechanism of which remains to be unraveled.

In recent years it became evident that gene expression is under the control of circadian rhythmicity (Kloppstech, 1985; Giuliano et al., 1988; Nagy et al., 1988), again at various levels. Transcription of many light-regulated nuclear genes coding for thylakoid membrane proteins is under the influence of circadian oscillations, which control the time of appearance of mRNAs for individual proteins (Giuliano et al., 1988; Taylor, 1989). Coordination of the endogenous oscillator(s) and the environmental factor light is achieved via the various photoreceptors, a process in which the role of phytochrome is predominant (Tavladoraki et al., 1989). Later during development and under light stress, blue light and UV-A receptors presumably become more important (Mullet, 1988). Translation of thylakoid membrane proteins on cytosolic ribosomes follows immediately after transcription of their genes (Adamska et al., 1991).

Furthermore, with the use of heat-shock treatments to achieve synchronization of the clock, it was found that greening is also under circadian control in pea (Otto et al., 1992) as well as in barley (Beator et al., 1992). Accumulation of mRNA for the light-harvesting protein, of its translation product, and of Chl *a* and *b* occurred in parallel but in variable amounts throughout the day. Whereas the accumulated amount of Chl *a* varied by a factor of 2, that of Chl *b* oscillated around 10-fold throughout the day. The mechanism, however, remained unknown, since it was possible that either the capacity for synthesis of Chl's varied during the day or, alternatively, that Chl's were stabilized by integration into the accumulated apoproteins. Therefore, we studied circadian regulation of Chl synthesis. In this publication we provide evidence for the existence of both mechanisms: stabilization of Chl by association with apoproteins and regulation of the capacity of synthesis of Chl's via δ-ALA.

MATERIALS AND METHODS

Plant Growth

Barley seeds (*Hordeum vulgare* cv Apex) were sown on vermiculite and grown in darkness at 25°C for 5 to 7 d at 90% RH. Starting at 9 h after sowing, plants received a 1-h heat shock of 40°C every 24 h for 5 d; thereafter, the temperature was held constant at 25°C. For illumination,

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* Corresponding author; fax 49-511-7623992.

Abbreviations: δ-ALA, δ-aminolevulinic acid; Cab, mRNA for LHC II protein; ELIP, early light-inducible protein; LHC II, light-harvesting Chl *a/b*-binding protein of PSII; PCOR, NADPH-protochlorophyllide oxidoreductase.

plants were exposed to fluorescent "daylight" of 15 W/m^2 for the indicated times, frozen in liquid nitrogen, and stored at −70°C.

RNA Isolation and Dot-Blot Analysis

RNA isolation, poly(A)$^+$ RNA selection, dot-blot analysis, and ^{32}P-labeled cDNA probes used were as described previously (Otto et al., 1992). Filters were cut into pieces of identical size and bound radioactivity was measured by Cerenkov counting. To determine background radioactivity, a piece of nucleic acid-free filter was also counted, and the value was subtracted from the resulting counts.

Pigment Extraction and HPLC Analysis

Pigment extraction and HPLC analysis was essentially as described (Beator et al., 1992) with the following modifications: the organic phase was extracted a second time with 20 mL of petroleum ether. Each petroleum ether phase was washed once with 20 mL of 4 M NaCl and twice with 10 mL of H_2O. Both petroleum ether phases were combined and then dried over Na_2SO_4.

Protein Extraction and Western Blots

Extraction of total proteins, SDS-PAGE, and immunodetection of LHC II, ELIP, and PCOR with alkaline phosphatase were as described (Adamska et al., 1991).

Pchlide Determination

Plants were exposed for 5 min to white light (15 W/m^2) and returned to darkness. Weighed material (approximately 2 g) from the apical 5 cm was ground under dim green "safety" light with sand in $NaHCO_3$-saturated 90% acetone. The leaf material was filtered and extracted quantitatively, and the total volume was adjusted to 10 mL/g fresh weight with 90% acetone. After a brief centrifugation, the cleared extract was assayed spectrophotometrically at 626 nm against 80% acetone. The amount of Pchlide was calculated using 31.1 L $mmol^{-1}$ cm^{-1} as the millimolar extinction coefficient according to Kahn (1983).

δ-ALA Determination

Two hours prior to illumination, the apical 5 cm were cut in the dark and placed in 80 mM levulinic acid, 10 mM KH_2PO_4, pH 7.2, or in 10 mM KH_2PO_4, pH 7.2, as a control. To facilitate uptake of the drug, segments were ventilated by a gentle stream of air during preincubation and illumination. Weighed segments of frozen material were homogenized in 1 N TCA, 1% SDS and centrifuged for 10 min at 65,000*g* at 4°C, and the cleared lysate was neutralized with an equal volume of 0.5 M NaH_2PO_4, pH 7.5, and condensed with 50 μL of ethylacetoacetate by boiling for 10 min in a water bath. The cooled extract was treated with an equal volume of modified Ehrlich's reagent (0.2 g of *p*-dimethylaminobenzaldehyde, 8.4 mL of acetic acid, 1.6 mL of 70% perchloric acid, freshly prepared before each determination), centrifuged for 5 min, and assayed spectrophotometrically at 553 nm. The amount of porphobilinogen formed from δ-ALA was calculated using 7.45×10^4 L mol^{-1} cm^{-1} as the extinction coefficient (Harel and Klein, 1972).

RESULTS

In previous publications (Beator et al., 1992; Otto et al., 1992), a constant time of illumination had been used to study accumulation of mRNA, protein, and pigments in etiolated plantlets that were synchronized by cyclic heat-shock treatments in the dark. This time period was chosen arbitrarily. However, we wanted to know whether identical or different time courses of greening would be achieved at the circadian maxima and minima of Cab mRNA, protein, and pigment accumulation. Therefore, greening was allowed to proceed for up to 8 h at the circadian maximum and circadian minimum of Cab mRNA levels. Representative data obtained with this experimental approach are presented in Figures 1 and 2. All data presented refer to these circadian times: the circadian maximum was determined to occur at 16 h and the circadian minimum at 24 h after the last heat-shock treatment (Beator et al., 1992).

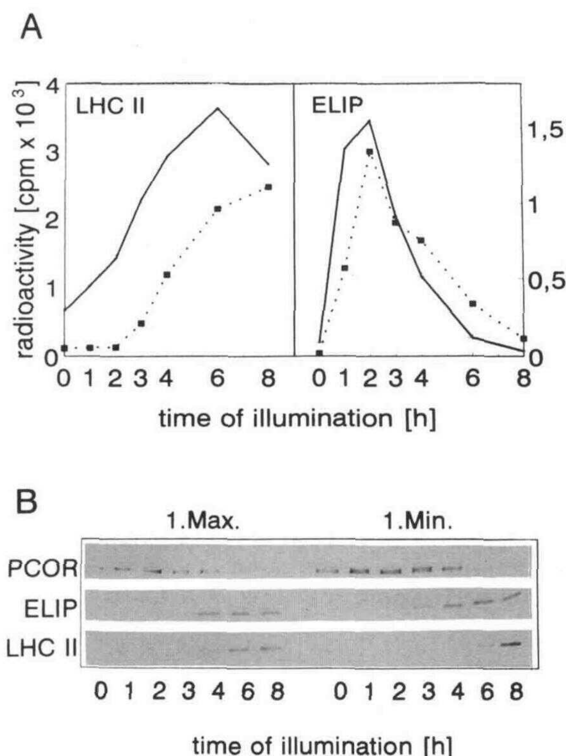

Figure 1. Circadian control of greening capacity: kinetic analysis of mRNA and protein accumulation. Five-day-old etiolated, cyclically heat-shocked barley plants were exposed at different circadian times to 15 W/m^2 of fluorescent light and harvested at the indicated times. —, First maximum (16 h after the last heat shock); ■···■, first minimum (24 h after the last heat shock). A, Poly(A)$^+$ RNA was isolated, blotted onto nylon filters, and hybridized to the indicated radioactively labeled cDNAs. Specific filter-bound radioactivity was measured by Cerenkov counting. B, Equal amounts of total protein extracts were assayed by western blot analysis for the proteins indicated.

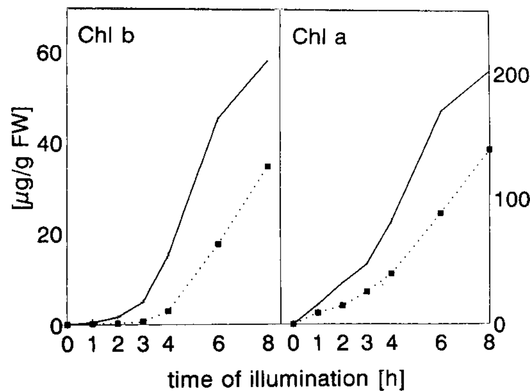

Figure 2. Circadian control of greening capacity: kinetic analysis of pigment accumulation. Five-day-old etiolated, cyclically heat-shocked barley plants were exposed at different circadian times to 15 W/m^2 of fluorescent light and harvested at the indicated times. ——, First maximum (16 h after the last heat shock); ■· · ·■, first minimum (24 h after the last heat shock). Pigments were isolated and analyzed by HPLC as described. Results are the average of two independent experiments.

When cyclically heat-shocked plantlets were transferred into the light at the circadian maximum, an immediate and almost linear increase in the level of Cab mRNA was observed during a period of 6 h; thereafter, the Cab mRNA levels started to decline (Fig. 1A). Illumination at the circadian minimum differed significantly: there was a lag of 2 h before Cab mRNA levels started to increase. This increase, however, occurred almost in parallel to that observed during the circadian maximum, so that after 8 h of illumination the mRNA levels were almost equal in both cases. Similarly, the level of ELIP mRNA increased more rapidly at the circadian maximum and also reached slightly higher values. The peak areas in both cases are almost equal, since the level of ELIP mRNA stayed elevated for a longer period of time when illumination took place during the circadian minimum. It cannot be decided whether this effect is due to an altered half-life of ELIP mRNA or whether transcriptional activity of the corresponding genes stayed elevated for a longer period of time (Fig. 1A).

In accordance with these findings, LHC II was already detectable in western blots after 4 h of illumination at the circadian maximum of Cab mRNA levels, whereas a comparable protein level was reached only after 6 h when illumination took place during the minimum (Fig. 1B). Western blots developed with the antibody for ELIP detected the protein after 3 h in both cases. The maximal ELIP level was achieved after 6 to 8 h. In contrast, levels of PCOR were higher when illumination occurred during the circadian minimum than during the circadian maximum; the PCOR obtained during the minimum appears to consist of two comigrating bands (Fig. 1B).

When accumulation of Chl's *a* and *b* was determined, circadian differences were obtained that were similar to those found for the changes in the Cab mRNA and the corresponding protein levels (Fig. 2); the kinetics of accumulation were somewhat different, however. The pigments accumulated at relatively low rates during the first 3 h. When plants were illuminated during the circadian maximum, the amounts of Chl's *a* and *b* accumulated at maximal rates between 3 and 6 h of illumination; thereafter, the rate of increase slowed down to some extent. This retardation of accumulation occurred in parallel to that of Cab mRNA (Fig. 1A). The levels of Chl *a* were about 3- or 4-fold higher than those of Chl *b*. In contrast, when plants were illuminated during the circadian minimum, accumulation of both Chl's started later but continued linearly throughout the period of measurement. However, there was a pronounced difference in the accumulation of Chl's *a* and *b* during the first 4 h of illumination: whereas Chl *a* increased, albeit at a much slower rate than between 4 and 6 h, there was almost no increase in the rate of accumulation of Chl *b* during the first 3 h. These differences in the time courses obtained during the circadian maximum and minimum, respectively, caused differences in the ratios of Chl levels during illumination (Fig. 3). Although ratios for Chl *a* between maximum and minimum remained constant at a level of 2 throughout the period of illumination, the ratio for Chl *b* went through a pronounced maximum after 3 h of illumination (Fig. 3).

To understand more closely the mechanism by which the difference in Chl accumulation occurs, we wanted to find out the rate-limiting step in Chl accumulation during greening, which is under circadian control. Because in angiosperms Chl's are formed from Pchlide via a light- and NADPH-dependent reaction and because light is the determining factor during greening (Griffiths, 1991), we analyzed the levels of Pchlide during a period of 4 to 48 h after the last heat shock. There was almost no difference between the times of circadian maxima and minima (data not shown). Similarly, formation of Chlide after transfer to saturating light flashes and regeneration of Pchlide was only slightly

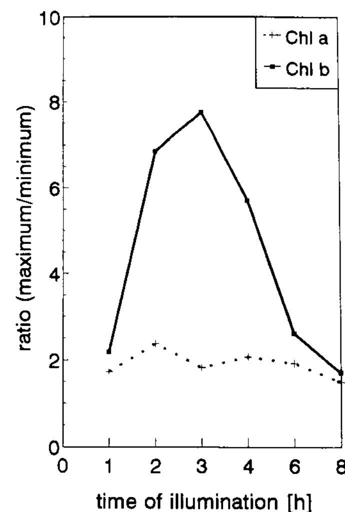

Figure 3. Development of the maximum/minimum ratio of Chl's during greening. Plantlets were exposed to light starting from either the circadian maximum or the circadian minimum. For each time point, the ratio of Chl content determined at the maximum and minimum, respectively, for both Chl's was calculated. Data are the average of two independent experiments.

different at the maxima and minima of Cab mRNA accumulation (data not shown). Also, at longer periods after return to darkness following 5 min of illumination there was no pronounced difference in the level of Pchlide (Fig. 4). Therefore, in our opinion, the differences in Chl formation cannot be explained sufficiently by the mechanism of phototransformation of Pchlide to Chl or by varying levels of either free or enzyme-bound Pchlide (Griffiths, 1991).

It is very well documented that synthesis of Chl is regulated at the level of δ-ALA synthesis and that this step is controlled by phytochrome (Masoner and Kasemir, 1975; Rüdiger and Schoch, 1988; Beale, 1990), which in turn has been found to set the phase of the clock (Tavladoraki et al., 1989). Therefore, the levels of δ-ALA were estimated throughout the day after treatment with levulinic acid at a concentration of 80 mM. This concentration of levulinic acid does not cause any toxic effects as far as δ-ALA formation is concerned (not shown). The treatment prevents condensation of δ-ALA by inhibition of δ-ALA dehydratase (Harel and Klein, 1972) and therefore blocks the flow of δ-ALA into the pathway leading to tetrapyrroles; this results in accumulation of δ-ALA from its precursors (Kannangara et al., 1988).

Under these conditions, it was possible to show that δ-ALA accumulates to more than 2-fold higher levels after 4 h of illumination in the circadian maximum compared with that achieved at the minimum (Fig. 5). Accumulation occurred with a 1-h lag when started in the circadian minimum. To find out whether this difference is also under circadian control, the illumination in the presence of levulinic acid was started at various times after the last synchronizing heat shock. Figure 6 shows that the maximum of δ-ALA accumulation coincides with the circadian maximum of Cab mRNA. This reaction appears to dampen out rather rapidly, however; a second peak of δ-ALA accumulation can be demonstrated to appear 36 h after the last heat shock.

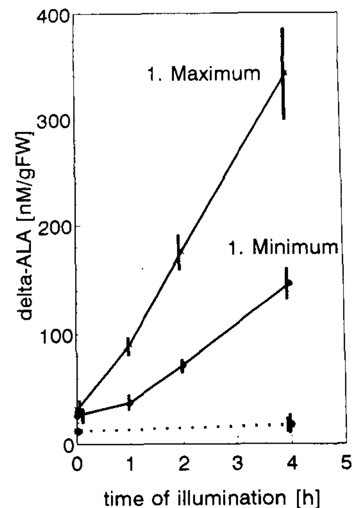

Figure 5. Circadian control of greening capacity: kinetic analysis of δ-ALA accumulation. Levulinic acid-treated segments were exposed at different circadian times to 15 W/m^2 of fluorescent light and harvested at the indicated times. Two hours prior to illumination, the plants were cut and 5-cm segments were placed into 80 mM levulinic acid, 10 mM KH_2PO_4, pH 7.2, or in 10 mM KH_2PO_4, pH 7.2 (controls). Data are the average of two independent experiments with at least four independent determinations per time point. SE values are indicated as bars. ···, Controls for maximum and minimum.

DISCUSSION

Since 1985, findings have accumulated indicating that circadian rhythmicity plays an important regulatory role during gene expression and differentiation of higher plants. This report adds some more information to support this view. It is well known that neither the Chl's nor their apoproteins are stable in the free, unbound form (Apel and Kloppstech, 1980; Otto et al., 1992). These observations do not support a priori the possibility that the synthesis of either one of the partners is regulated while the counterpart is synthesized continuously without control, being stabilized by complex formation. Although possible in principle, this assumption

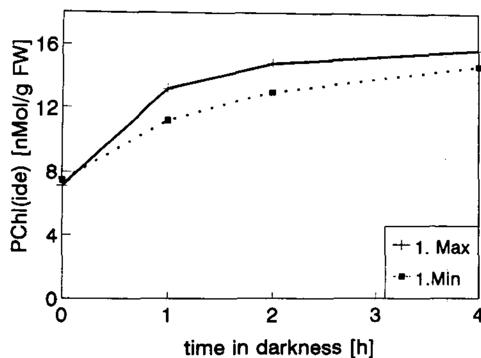

Figure 4. Pchlide regeneration in the dark after 5 min of illumination. Five-day-old etiolated, cyclically heat-shocked barley plants were exposed at different circadian times to 15 W/m^2 of fluorescent light for 5 min, returned to darkness, and harvested at the indicated times. The amount of total Pchl(ide) in an acetone extract was estimated spectrophotometrically as described in "Materials and Methods." Data are the average of two independent experiments.

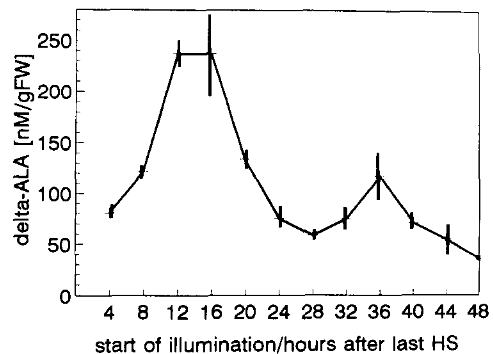

Figure 6. Circadian control of δ-ALA accumulation after cyclic heat-shock treatment. Levulinic acid-treated segments were placed under 15 W/m^2 of fluorescent light for 2 h at the indicated times after the last heat shock for 2 consecutive days. Plant material was frozen in liquid nitrogen and δ-ALA levels were estimated as described. Data are the average of two separate experiments with three or more independent determinations. SE values are indicated as bars.

ignores the fact that organisms normally do not seem to allow waste of energy. The coordination of these processes by light itself seems feasible, because one of the last steps in Chl biosynthesis is light dependent, as is initiation of transcription of light-inducible genes (Gallagher and Ellis, 1982). This interpretation does not take into account that light control acts primarily via phytochrome by setting the clock (Nagy et al., 1988; Tavladoraki et al., 1989). The extent to which light intensity itself interferes with the various levels of gene expression is not so well understood. Only recently it was found that light intensity selectively interferes with transcriptional activity of individual genes (Oelmüller, 1989; Adamska et al., 1992).

Circadian control of light-induced thylakoid membrane formation is also revealed by the different kinetics of greening, which have been shown here to occur at the times of circadian maximum and minimum (Figs. 1 and 2). The lag observed at the minimum is very similar to that found during greening of etiolated plants after transfer from the dark into the light (Kasemir et al., 1973). The fact that this lag could be abolished by a short preillumination pulse a few hours prior to illumination can now also be interpreted as a result of circadian control. This view is supported by the finding that greening during the circadian maximum occurs without this lag, thus indicating that, after transfer from the dark into the light, phase setting of the clock occurs in phase with the oscillations that, during growth in the dark, had been originated or coordinated by the cyclic heat-shock treatments (Beator et al., 1992; Otto et al., 1992).

Findings published recently indicate that there is coordinated control of LHC II and Chl synthesis (Beator et al., 1992). Due to the fact that pigments are stabilized after their integration into the protein, our data were not sufficient to argue that control of both pigment and protein synthesis is synchronized by the circadian oscillator. On the contrary, the extent (a factor of 8) to which Chl *b* oscillated between maximum and minimum as compared with Chl *a* (a factor of 2) indicated that, in addition to having a higher rate of synthesis, it also exhibits stabilization by its binding to the apoprotein (Fig. 1B).

For the regulation of Chl synthesis, we expected a control at the level of Pchlide, especially since the levels of PCOR mRNA are under control of light. Quite a number of experiments to show this type of regulation failed, however. A very predominant control over the tetrapyrrole pathway in higher plants is exerted at the level of δ-ALA (Masoner and Kasemir, 1975). Although not established thus far, the long-known interference of the photoreceptor phytochrome with Chl synthesis at the level of δ-ALA indicated the possibility that the circadian regulation of Chl might occur at this level. Accumulation of δ-ALA in the presence of levulinic acid offered the possibility of measuring δ-ALA levels. Indeed, these were found to oscillate by a factor of 2 during the day, indicating that at least one of the enzymes of the pathway leading to synthesis of δ-ALA is under circadian control. Preliminary experiments at the level of transcripts made it unlikely that glutamate 1-semialdehyde aminotransferase could play this role (data not shown). We would like to suggest that glutamyl-tRNA-reductase might be the controlled entity; a similar opinion has been put forward by Rüdiger and Schoch (1988).

These findings, however, do not explain the tremendous variation in the level of Chl *b*. This could be due to the fact that a threshold level of Chl *a* has to be reached prior to synthesis of Chl *b*. We prefer, at present, the hypothesis that Chl *b* is stabilized by its binding to the LHC II apoprotein, which is synthesized at higher rates during circadian maxima.

In conclusion, our data indicate that the circadian control of Chl accumulation after synchronization by heat shock is of dual origin. The rate of synthesis of δ-ALA is subject to circadian control, accounting for the approximately 2-fold variation observed in Chl *a* accumulation (Figs. 5 and 6). Additionally, helping to explain the time course of Chl *b* accumulation, the availability of LHC II apoprotein appears to be the limiting factor under the chosen conditions. Because LHC II binds about 50% of Chl *b* in green plants (White and Green, 1987), this would be a plausible explanation. We assume that as soon as a sufficient amount of Cab mRNA has accumulated during illumination at the circadian minimum allowing for a significant synthesis of LHC II apoprotein, Chl *b* is stabilized; as a consequence, the ratio between maximum and minimum for Chl *b* drops to 2- to 3-fold. This is the value also observed for Chl *a*, which can be explained by the circadian variation of δ-ALA synthesis rate.

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