The Effect of Heat Shock on Morphogenesis in Barley

Coordinated Circadian Regulation of mRNA Levels for Light-Regulated Genes and of the Capacity for Accumulation of Chlorophyll Protein Complexes

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ABSTRACT

The effect of daily heat-shock treatments on gene expression and morphogenesis of etiolated barley (Hordeum vulgare) was investigated. Heat-shock treatments in the dark induced shortening of the primary leaves and the coleoptiles to the length of those in light-grown plantlets. In addition, the mRNA levels of the light-induced genes that were investigated were raised under these conditions and showed distinct oscillations over a period of at least 3 d. While the mRNA levels for chlorophyll a/b binding protein (LHC II), plastocyanin, and the small subunit of ribulose-1,5-bisphosphate carboxylase had maxima between 8 and 12 PM (12-16 h after the last heat-shock treatment), the mRNA levels for thionin oscillated with a phase opposed to that of LHC II. Etiolated barley, the circadian oscillator of which was synchronized by cyclic heat-shock treatments, was illuminated for a constant interval at different times of the day; this led to the finding that greening was fastest at the time when the maximal levels of mRNA for LHC II were also observed. Whereas accumulation of chlorophyll a during a 4-h period of illumination oscillated by a factor of 3, chlorophyll b accumulation changed 10- to 15-fold. Similarly, accumulation of LHC II was highest when pigments accumulated maximally. Hence, greening or, in other words, thylakoid membrane assembly is under control of the circadian oscillator.

Light controls plant development at various levels of gene expression in the nuclear-cytosolic compartments as well as in the plastid. Light-enhanced or light-repressed activity of nuclear-coded plastid proteins is regulated basically at the level of transcription (12). In addition, other consecutive levels of regulation exist (3). In the first instance, the photoreceptor phytochrome is involved in the perception of light, leading to the expression of nuclear-coded genes for the plastid (2). Other, eventually subordinated receptors such as blue-light (18) and near UV-light receptors (21) also play a role.

The study of gene expression in etiolated plants has many advantages for unraveling the mechanisms of the expression of light-regulated genes, particularly for investigations of the action of phytochrome. Originally, it was assumed that phytochrome acts primarily at the level of transcription (2, 22). After the discovery of circadian regulation of mRNA levels (19), evidence accumulated suggesting that transcription of light-regulated genes is primarily under the control of the circadian clock (14) and that signal perception occurs via phytochrome (27). Moreover, the phase of the endogeneous clock can be shifted via phytochrome (27). Thus, a primary synchronization between the circadian rhythm of gene expression and the external factor light can be achieved. These findings imply an interaction of phytochrome with the mechanism of the circadian oscillator itself. The signal chain(s) that leads from the receptor(s) via the endogeneous clock mechanism to the transcription of genes is not yet known.

To understand more closely the potential effect of circadian rhythm on gene expression and morphogenesis, we studied the influence of external signals (zeitgeber) other than light. Of these, temperature shifts appeared well suited because alterations in the ambient temperature affect the phase of the circadian rhythm (26). The positive effect of temperature oscillations on development was shown in experiments in which it was found that plants grown under constant environmental conditions do not develop properly, whereas a diurnal fluctuation of temperature under otherwise constant conditions has a normalizing effect on plant development (16).

In previous studies (20), we have shown that HS treatments and diurnal temperature shifts during emergence of pea seedlings in the dark cause morphogenetic changes that are very similar to those induced by far-red light: plumulae are developed, the hooks opened, and the stems shortened. These "photomorphogenic" characteristics are accompanied by elevated levels of nuclear-coded, light-inducible mRNAs for LHC II and SSU. ELIPs are another group of light-regulated genes (15): the regulation in their steady-state mRNA level remains entirely light dependent in etiolated pea seedlings whose circadian oscillations were entrained by temperature cycles. This type of regulation of ELIP mRNAs, in contrast with those of LHC II and SSU, demonstrates specificity of the temperature effect with regard to its partial

1 This work was supported by Deutsche Forschungsgemeinschaft, Bonn, FRG.

2 Abbreviations: HS, heat shock; LHC II, chlorophyll a/b binding protein; SSU, small subunit of Rubisco; ELIP, early light-inducible protein.
substitution for light. We have found that in spinach the levels of the mRNAs for most of the thylakoid membrane proteins that were investigated oscillate during the light-dark cycle (A. Schneiderbauer, R. Herrmann, and K. Kloppsteck, unpublished data). Based on this, it was proposed that other light-inducible genes might also be under the control of a circadian oscillator after heat treatments (20).

Monocots differ in many aspects of their development from dicots. Development of leaves takes place in the dark, although no Chl is formed. In etiolated monocots, phytochrome seems to play a much more pronounced role during light perception than in most of the dicots that have been studied. Furthermore, cells are arranged along a linear gradient of development, allowing for the analysis of developmental aspects of gene expression (29). We wished to determine whether heat treatments can act as a zeitgeber in monocots, as they apparently do in dicots, and examined this phenomenon in barley (Hordeum vulgare), since this species is well suited for the investigation of developmental in combination with circadian aspects of gene expression (29).

MATERIALS AND METHODS

Plant Growth and Statistics

Barley seeds (Hordeum vulgare cv Aramis or, in later experiments, cv Apex) were sown on vermiculite and grown in darkness for 5 to 8 d at a RH of 90%. Temperature was either held constant at 25°C (etiolated) or a daily HS of 40°C was applied between 7 and 8 AM (cyclic HS). Plants were harvested in the dark or after an illumination period of 4 h at 15 W/m² in fluorescent “daylight,” frozen in liquid nitrogen, and stored at −70°C. All experiments were carried out at least in duplicate and with 30 to 60 plantlets per data point.

RNA Isolation

Six grams of plant material (30 to 60 leaves) were ground in liquid nitrogen and extracted as described (20). Poly(A)+ RNA was selected by oligo(dT) chromatography (3).

Dot Blots

Dot blotting and hybridization conditions were essentially as described (20). The mRNA contents were adjusted to equal concentrations after measurement by spectrophotometer at 260 nm using a 50-μL microcuvette. With equal volumes of these solutions, up to 10 identical replica filters were prepared and used for dot blotting. After autoradiography, signals linear in intensity with exposure time (A_dual < 0.8) were scanned at 600 nm for quantification. Some of the filters were stripped and rehybridized with another probe to ensure reproducibility of the data.

The probes used were homologous cDNA clones for LHC II, SSU, thionin, plastocyanin, and ubiquitin that were kindly provided by Dr. K. Gausing, Aarhus (4). ELIP mRNA levels were determined using the cDNA encoding a small ELIP (15). HSP No. 138 codes for a 17-kD cytosolic HS protein of barley (E. Kruse and Z.-L. Liu, unpublished data).

Pigment Extraction

Leaf material (1.5–2 g) was harvested from the apical 6.5 cm of the primary leaf, frozen, and ground in acetone with sand. The material was extracted three times with 10 mL of acetone and filtered. The pigments were transferred into 25 mL of petroleum ether in a separatory funnel, and the pigment-containing phase was washed once with 20 mL of 4 M NaCl and twice with 20 mL of H₂O. The petroleum ether was dried with Na₂SO₄ and evaporated under vacuum, and the pigments were dissolved in 1 to 1.5 mL of acetone.

HPLC Analysis

HPLC analysis of pigments was performed as described by Humbeck et al. (17) on a Kontron (München, FRG) HPLC system consisting of Pump 420, Gradient former 425, UV/Visible Detector 430, and Data System 450. Pigments were identified by their absorption spectra recorded during the run (Chl a and Chl b) or by injecting purified β-carotene and luteine and comparing their respective retention times. Peaks at a detection wavelength of 445 nm were integrated by the Data System 450, and the relative units produced by the integrator were standardized according to the fresh weight and volume of individual samples. Samples were loaded on a 5-μm RP18 column with a Rheodyne solvent valve equipped with a 20-μL loop. The pigments were separated by a ternary gradient system: 90% solvent A (acetonitrile:methanol, 75:25%; v/v) was blended with 10% solvent B (water). Solvent A increased linearly to 100% after 40 min, was held constant for 10 min, and decreased to 90% during the last 10 min of the run. The flow rate was 1 mL/min. Solvents were degassed by a stream of helium.

Protein Extraction and Western Blots

Weighed leaves (2 g) were extracted in sample buffer (56 mM Na₂CO₃, 0.1 M DTT, 2% SDS, 10% glycerol in 10 mL/g of leaf material) with a potter, heated at 65°C for 20 min, and boiled for 5 min. The clarified supernatant (10,000g for 10 min) was applied to 12% polyacrylamide gels. Electrophoresis, transfer of proteins to nitrocellulose, and visualization of LHC II protein by alkaline phosphatase were as described (1).

RESULTS

The Effect of Heat Treatments on Morphology of Etiolated Barley Seedlings

In comparison with the pronounced differences in the development of dicots, the growth of etiolated barley primary leaves is only slightly different from that of light-grown plantlets (25). However, the leaf blades remain enrolled and are enclosed by the coleoptile for a longer time. The coleoptiles grow about twice as long in comparison with normally developed green plants. After heat treatments, the appearance of etiolated plantlets is changed in a characteristic manner toward that which is typical for light-grown plants. The leaves are partially unrolled. Furthermore, the length of the blade of the first leaf (Fig. 1A) is reduced compared with
that of etiolated plantlets or is even slightly shorter than that of light-grown seedlings. Elongation of control and heat-
shocked leaves ceases after about 7 d.

The effect of HS on the growth of coleoptiles is even more pronounced (Fig. 1B). Following light or heat treatments,
longitudinal growth stops after 3 d. In the etiolated controls,
elongation of coleoptiles continues during the 4th d, resulting
in doubling of the final length of the coleoptiles.

Circadian Oscillations in mRNA Levels for Nuclear-coded
Plastid Proteins

The heat-induced effects on plant morphology outlined
above indicate that heat is perceived to some extent as a
substrate for a light signal. Because light pulses induce
circadian oscillations of mRNA levels in etiolated plants (27),
we analyzed the effect of HS treatments on the levels of
several different mRNAs for nuclear-coded plastid proteins
during several consecutive days (Fig. 2) by dot blot. This was
possible, since for most of the probes used, like LHC II, SSU,
thionin, and plastocyanin (4), as well as ELIP (15) and HS
proteins (E. Kruse and Z.-L. Liu, unpublished), it had been

shown that only a single major band was obtained in northern
blots. The mRNA for the apoprotein of LHC II oscillates
after the last heat treatment for at least 3 d under constant
conditions in the dark (Fig. 2). The oscillations are dampened
during this period, but the maxima remain clearly evident,
and, additionally, their position is constant throughout
the experiment. The maximal levels are reached about 16 h
after the last HS treatment, which was from 7 to 8 AM. A similar
curve has been obtained for plastocyanin. The amplitude of
these oscillations changed by a factor of 4; in this case, the
maximum was reached at 8 PM, or 12 h after the last HS
treatment (Fig. 2); however, at days 2 and 3 the appearance
of the curve was biphasic, and the second peak occurred at
12 PM.

In comparison with the very low levels of mRNA for LHC II
in dark-grown controls, those for SSU are relatively high
in dark-grown barley (3). For this mRNA, a broad biphasic
oscillation is obtained with minima at 8 AM, which is the time
when the daily heat treatments ended (Fig. 2).

In heat-treated pea, the levels of ELIP mRNA do not
increase following HS (20). In barley, however, a high level
of ELIP mRNA accumulates 12 h after the last HS. A small
amount of this transcript is detectable during the 2nd d at
the expected time. The mRNA was undetectable on the 3rd
d. This indicates a transient, heat-induced enhancement of
ELIP mRNA levels (Fig. 2) unlike those of LHC II, plastocy-
ain, and SSU mRNAs (Fig. 2), which show more persistent,
although dampened, oscillations.

A question of interest was to what extent the levels of LHC II
and SSU mRNAs induced by HS are comparable with
those obtained by light. In Figure 3, the mRNA levels ob-
tained at the first maximum after the last HS are compared
with the values obtained with a sample taken from light/dark-grown plantlets at noon, when these mRNAs are max-
imal. This analysis was also performed on etiolated plantlets
after 4 h of illumination, when ELIP is expressed maximally.
When the maximal values in the respective light-treated
controls are set to 100%, the mRNA levels of ELIP, LHC II,
and SSU in the heat-shocked, etiolated plantlets reach 25,
10, and 30% of the corresponding maximum, respectively.

Circadian Oscillations of mRNAs for Cytosolic Proteins

As expected, the heat treatment induces a tremendous
increase in the level of mRNA for HS proteins. In Figure 4
this heat-induced level can be seen for the mRNA of a small
cytosolic HS protein of 16 kD in the first sample taken
immediately after the last HS treatment. In accordance with
published data (28), the level of this mRNA drops during
recovery within less than 4 h to almost the background level.
Surprisingly, the steady-state level of this mRNA also seems
to oscillate in a circadian manner. Under constant conditions,
the mRNA level increases again around 16 h after the HS
and once more at the corresponding circadian time of the
second day in the absence of any inducing HS. The tiny
maxima coincide with the peak levels of mRNA for LHC II.

The mRNA levels for ubiquitin, which is considered a low
molecular mass heat-stress-inducible protein (7, 24), are
rather low and did not show clearcut oscillations during
the experiment (Fig. 4). This mRNA can be considered an endog-
Circadian Oscillations in the Capability for Accumulation of Pigments and Light-Harvesting Chl a/b Protein

In pea, we observed that etiolated plantlets showed accelerated light-induced accumulation of pigments and thylakoid membrane proteins after heat treatments (23). Under identical experimental conditions with regard to the time of heat treatments and beginning of illumination, no such accelerating effect could be observed in barley plantlets. There was, however, a remarkable difference with respect to the position of maxima in the LHC II oscillation induced by the HS in the two species. In pea, the maximum in the level for LHC II mRNA was reached at noon, whereas the maxima in barley were obtained at midnight, indicating that in pea the peak of this circadian rhythm is positioned to the beginning of the subjective light phase. The same treatment in barley shifts the phase by about 12 h relative to the maximum observed in pea. Therefore, we studied greening of etiolated barley at different times of the day after cyclic HS treatment. This was done by exposing barley plantlets at 4-h intervals to 4 h of light during the 2 consecutive days following the last HS treatment. Immediately thereafter, the plantlets were frozen.
in liquid nitrogen. The extracted plastid pigments were separated by HPLC and quantified by integration of the peaks. In Figure 5, it is shown that the amounts of β-carotene and luteine that can be extracted from the plantlets per unit of fresh weight after 4 h of light oscillate only slightly during these 2 d. In contrast, the amounts of Chl a and b that accumulated during the 4-h period of illumination vary considerably throughout the day. The maximal amounts of Chl a and b are obtained when the greening is started at midnight, and the minima are found at 8 AM, indicating that the capacity for Chl synthesis and the maximal levels of mRNA coding for LHC II occur in parallel (see Fig. 2 for comparison). Preliminary data indicate that, in addition, the amounts of LHC II mRNA that accumulate during the 4-h light periods vary in parallel. The amount of Chl a accumulated during 4 h changes by a factor of 3, whereas that of Chl b fluctuates by about a factor of 10 to 15.

In parallel with the higher rate of accumulation of Chl during a 4-h illumination period at 16 h after the HS, the apoprotein of the LHC II complex also accumulates to a higher level than at 4 h after the HS. This is in agreement with the high levels of mRNAs for LHC II that are already present at the beginning of illumination at this particular circadian time (Fig. 2). Figure 6 represents the typical result of a western blot performed with samples collected throughout the day at 4-h intervals after 4 h of illumination. The highest accumulation of LHC II occurs when the illumination period starts at 24 h (± 4 h) circadian time.

**DISCUSSION**

Heat treatments evoke morphogenetic changes in etiolated plantlets that are very similar to those known to occur in response to light. When caused by light they are described as photomorphogenesis; many of these light-induced effects are known to be mediated via phytochrome. Here we show that HS treatments cause effects very similar to those stimulated by light. The most prominent and typical effects of HS on morphogenesis in barley are a reduction of the elongation

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**Figure 4.** Oscillation of mRNAs for nuclear-coded, nonplastid-localized proteins. Replica filters obtained with the samples described in the legend to Figure 2 were hybridized to the following cDNAs: HSP No. 138, ubiquitin, thionin. Scanning is described in the legend to Figure 2, and the corresponding autoradiograms are represented on top of the individual panels. The light dotted line in the thionin panel shows the LHC II fluctuations from Figure 2. The mRNA levels for each of the probes in the etiolated control plantlets are also represented (●—●—●).

**Figure 5.** Circadian changes in the capacity to accumulate chloroplast pigments. Barley seeds were grown under the cyclic HS regimen as described. At day 5, the HS was omitted and the plants were transferred to fluorescent daylight of 15 W/m² for exactly 4 h at the indicated times during 2 consecutive days. Pigment extraction and HPLC analysis were performed as described, and the maximum amount of pigments accumulated during the experiment was set to 100% for each pigment as indicated.
of the primary leaves and of the coleoptile, closely resembling the typical photomorphogenetic effect of light. The signal chain leading from recognition of the HS to changes in morphogenesis is entirely unknown.

The argument that the morphogenetic effects could be explained assuming that heat accelerates processes occurring normally during etiolation can be excluded based on the observation that there are a number of concomitant morphological changes induced by either light or heat. These include the reduction of leaf and coleoptile elongation in the dark in barley, and of stem elongation in pea, whereas, in contrast, in the latter species the leaf area increases by a factor of 10. These photomorphogenetic traits are thought to be under the control of phytochrome. In addition, in the heat-treated etiolated plantlets mRNA levels for light-inducible proteins are elevated and oscillate in a circadian manner similar to those observed after light pulses (27). Another example of light-independent “photomorphogenesis” is the deti mutant of Arabidopsis thaliana described by Chory and Petö (8), which shows almost no signs of etiolation and possesses elevated levels of light-inducible proteins in the dark. It is not known if, in these plantlets, the corresponding mRNA levels oscillate as well. A release of morphogenesis by mutation within a system for negative feedback on light-inducible gene expression has been postulated by the authors to explain the findings obtained with this mutant.

Light apparently exerts two different effects on the expression of light-inducible genes: induction of circadian rhythmicity (27) in mRNA levels per se and a longer-lasting enhancement of transcript levels (3, 23). Under illumination, these two different effects are not experimentally separable. Heat, on the other hand, leads primarily to the induction of circadian oscillations in mRNA levels but not to a longer-lasting (e.g. 100-fold or more above background) increment in the levels of light-inducible genes (see Fig. 3). Using the methods of promoter deletion and expression of the constructs in transgenic plants, Nagy and his group (11) found that there are indeed two different domains responsible for the enhancement and the basic circadian transcription pattern. Whereas light affects both regions, we propose that heat might affect primarily the region that is under control of the circadian oscillator. Therefore, the periodic heat treatments described here offer an experimental approach with which to dissect this complex type of gene regulation. The coincidence between the circadian control of many light-inducible genes and of morphogenesis by both light and temperature may indicate that the few examples described here of circadian expression of genes after HS comprise only a small part of the genes which are affected. The findings might be summarized in the following hypothesis:

Heat → Clock mechanism → gene expression → morphogenesis

Figure 6. Circadian changes in the capacity to accumulate LHC II. Equal amounts of plant material were taken from the same samples described in Figure 5, and total SDS-soluble proteins were extracted, separated on a 12.5% polyacrylamide gel, and transferred to nitrocellulose membranes. The filters were probed with an anti-LHC II antibody. Immunoreactive LHC II bands were stained with an alkaline-phosphatase-conjugated secondary antibody (1).

An interesting finding is that the extent of greening as measured here by quantification of chloroplast pigments and the apoprotein of LHC II itself varies in a circadian manner in parallel with the oscillations of mRNA for LHC II. These data indicate that greening and hence chloroplast biogenesis is under circadian control after HS and/or light treatments. Similar findings on Chl accumulation induced via phytochrome have been observed earlier; however, the interpretation of these findings was different (13). Because the same result has not been observed in etiolated plantlets, it would be reasonable to assume that in etiolated plants the circadian clock does not oscillate or, if oscillating, is not under the synchronizing control of a circadian pacemaker but can be synchronized by HS (and/or light). A similar observation was made indirectly when it was found that the interval between preillumination and illumination had a high impact on the extent of greening (6). However, the interpretation that this is the result of interference of an external signal (heat or light) with a circadian oscillator is a new one. In our opinion, this finding on circadian regulation of greening (i.e. membrane and structure formation) has important implications for the understanding of chloroplast biogenesis. If a preillumination signal synchronizes the rhythm to subjective noon, the transient response observed directly after a preillumination light pulse would mainly reflect the activity of gene expression for light-inducible proteins that occurs during subjective afternoon. A red-light pulse initiates a circadian rhythm in LHC II mRNA accumulation with a transient response immediately after the pulse (27).

It follows that other aspects like stability of the thylakoids against external effects of high light or extreme temperatures will be under circadian control as is photosynthesis. This can be concluded from the fact that light-induced Chl accumulation after induction of rhythmicity by HS, especially that of Chl b, is under circadian control either directly or due to stabilization by the formation of protein-pigment complexes. The essential prerequisite, namely a circadian-regulated accumulation of LHC II, is present (Fig. 6). The maximum in the accumulation coincides very well with the maximal level of LHC II mRNA observed after an HS (Fig. 2). We (1) and others (9) have also shown that mRNA maxima concur with the maximal expression of the corresponding proteins. It follows that under normal light/dark cycles, the simultaneous accumulation of Chls and proteins guarantees the essential stabilization (3) of both components of the Chl-protein complexes. In the plastid compartment, this coordination might be achieved by translational control of protein synthesis via
the accumulating Chl, as has recently been suggested (10). Thus, the physiological significance of a circadian synchronization of membrane formation appears to be very high and could help to explain the well-established circadian rhythmicity of oxygen evolution.

Our data present additional evidence that the regulation of gene activity during normal light/dark conditions is a very complex event. This regulation includes a coordinated interlude at the various levels of gene expression that is governed by the circadian oscillator. The unraveling of this complex type of circadian regulation of gene expression will be important for the further understanding of plant development.

ACKNOWLEDGMENTS

We are very grateful to Dr. Kirsten Gausing, University of Aarhus, Aarhus, Denmark, for her generosity in providing us with the homologous probes to LHC II, plastocyanin, SSU, thionin, and ubiquitin.

LITERATURE CITED