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Regulation of 5-Aminolevulinic Acid (ALA) Synthesis in Developing Chloroplasts[1]

II. Regulation of ALA-Synthesizing Capacity by Phytochrome

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ABSTRACT

When dark-grown cucumber (*Cucumis sativus* L.) seedlings previously exposed to white light for 20 hours were returned to darkness, the ability of isolated chloroplasts to synthesize 5-aminolevulinic acid dropped by approximately 70% within 1 hour. The seedlings were then exposed to light, and the synthetic ability of the isolated chloroplasts was determined. Restoration of the synthetic capacity was promoted by continuous white or red light of moderate intensity. Intermittent red light was also effective. Blue and far-red light did not restore the synthetic capability. Blue light given after a red pulse did not enhance the effect of the red light. Far-red light given immediately after each red pulse prevented the stimulation due to intermittent red light. Restoration of the biosynthetic activity by *in vivo* light treatments was inhibited by cycloheximide indicating the requirement for translation on 80 S ribosomes for the *in vivo* light response. These findings suggest that the majority of the plastidic 5-aminolevulinic acid synthesis is under phytochrome regulation.

The synthesis of Chl in angiosperms is a light-dependent process (4, 5). This is so not only because the reduction of ring D (the conversion of Pchlide to Chlide) requires light, but light regulation also extends to the synthesis of Pchlide (14, 24). In dark-grown tissue, Pchlide synthesis is limited; but upon exposure to light, the Pchlide initially present is transformed to Chlide and then to Chl. After a variable delay, the 'lag phase,' a rapid synthesis of Pchlide takes place, which, in continuous light, is quickly converted to Chl (1, 17, 18, 21, 22, 26).

The lag phase of greening has been investigated in a number of tissues in a number of laboratories. In many cases it has been seen that the removal of the lag phase is under control of the phytochrome system, and that the phytochrome system somehow modulates the synthesis of ALA[2] in the early stages of greening (16, 17, 22, 23). Many other biochemical and cytological changes take place during the lag phase, which are also under the control of phytochrome (14, 24).

A great deal of interest has been funneled in recent years into the relationship between the phytochrome regulation of Chl accumulation and the phytochrome regulation of Pchlide reductase (EC 1.3.1.33) and of Chl *a/b* binding proteins (2, 10, 20). Exogenous ALA is rapidly converted to Pchlide, even in the dark, suggesting that the regulation of Pchlide synthesis by light takes place at the level of ALA synthesis (14, 24). Until recently, it has been generally thought that the phytochrome regulation is limited to the early stages of greening and that feedback inhibition of ALA synthesis by pchlide controls ALA synthesis later on (14). In the companion paper (12), we have examined the regulation of ALA synthesis in chloroplasts isolated from partially greened cucumber cotyledons and have found that feedback inhibition of ALA synthesis by Pchlide is not significant. However, we have observed that approximately two-thirds of the ALA-synthesizing capacity exhibited *in organello* is modulated by light/dark *in vivo* treatments, prior to chloroplast isolation. The light-dependent *in vivo* restoration of the ALA synthesizing capability has now been investigated further and shown to be phytochrome-mediated by the usual criterion of a red light-dependent response, which is inhibited when the red light pulse is immediately followed by a pulse of far-red light.

MATERIALS AND METHODS

Materials

Cycloheximide was purchased from Sigma. Other materials were obtained as described previously (12).

Methods

Light Treatments

Several illumination arrangements were used to achieve the desired combinations of photon flux density, light quality, and light duration.

For continuous illumination with white, red, or blue light, the source consisted of warm white, high output, Phillips fluorescent tubes. The intensity of white and red light was adjusted by means of neutral density filters. Red light was obtained by filtering white fluorescent light through one layer of red Plexiglas plus one layer of amber cellulose acetate. Blue light was obtained by filtering white fluorescent light through one layer of blue Plexiglas.

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[2] Abbreviations: ALA, 5-aminolevulinic acid; [H], reducing power consisting of 4 mM NADPH and 4 mM glucose-6-phosphate (15); LVA. levulinic acid; SAM, *S*-adenosyl-L-methionine; CHX, cycloheximide.

For intermittent red light experiments (2 min red pulses, separated by 10 min of dark or by 3 min of far-red plus 7 min of dark), the source consisted of three 15 W cool-white fluorescent tubes. The white light was filtered through one layer of amber Plexiglas and one layer of red Plexiglas (3). Photon flux density was approximately 3.0 $\mu E \cdot m^{-2} \cdot s^{-1}$.

For intermittent far-red light experiments, the light source consisted of five 300 W reflector flood lamps filtered through 12 cm of H_2O and two layers of far-red FRF acrylic plastic (3). In red-far-red photoreversibility experiments, the seedlings were moved by hand from one light source to the other.

Photon flux density measurements were taken with a LI-COR PAR meter.

Application of CHX to Dark-Treated Tissue

Cotyledons from tissue that had been dark-treated for 1 h were excised under a green safelight. Ten g of cotyledons were placed in a Petri dish 14 cm in diameter, containing 20 mL of 10 mM Na phosphate (pH 6.8) with or without the protein synthesis inhibitor. The tissue was vacuum-infiltrated and the vacuum released three times. The infiltration process lasted for a total of 10 min. The tissue was incubated for an additional 50 min in total darkness.

ALA Assays

ALA synthesis in isolated chloroplasts can be followed either in the dark with exogenous ATP and [H], an equimolar mixture of NADPH and glucose-6-phosphate (11), or in the light, without the addition of these cofactors, but relying on photosynthesis to produce the required ATP and NADPH (11). The final determination of ALA was according to Weinstein and Beale (25).

Pchlide Assay

Pchlide was extracted and assayed according to Castelfranco *et al.* (6).

RESULTS

We have previously observed (12) that ALA-synthesizing capacity in isolated chloroplasts greatly declines when etiolated cucumber seedlings that have been exposed to white light (60 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 20 h are returned to the dark, and largely recovers when the dark-treated seedlings are again exposed to continuous white light (12). The decline and recovery were complete in approximately 50 min (12). In the present study, the light-mediated *in vivo* restoration of the ALA-synthesizing capacity has been further examined.

Exposure of dark-treated seedlings to 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ white light increased ALA-synthesizing capacity in isolated chloroplasts by well over twofold. Six $\mu E \cdot m^{-2} \cdot s^{-1}$ of white light was almost as effective as 60 in bringing about restoration of the ALA-synthesizing capacity (Table I).

The red component isolated from the same white light source exerted nearly the same effect as the white light (Tables II and III), while blue light of similar intensity (1.2 or 3.9 $\mu E \cdot$

Table I. *Effect of Different Photon Flux Densities of White Light on the Recovery of the ALA-Synthesizing Capacity*

Twenty h greened seedlings were dark treated for 2 h. Chloroplasts were isolated either at the end of the dark treatment or after the seedlings were exposed for an additional 50 min to continuous white light of different flux densities. The isolated chloroplasts were incubated in the light (approximately 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) for 20 min with 10 mM LVA and 6 mM Glu to determine ALA synthesis. Other conditions were as indicated in "Materials and Methods." In this and subsequent tables, the entries are means and range of duplicate measurements.

In Vivo Illumination	ALA-Synthesizing Capacity
	pmol·mg protein^{-1}
Dark control	793 ± 23
White, 60 $\mu E \cdot m^{-2} \cdot s^{-1}$	2259 ± 46
White, 19 $\mu E \cdot m^{-2} \cdot s^{-1}$	2254 ± 49
White, 6 $\mu E \cdot m^{-2} \cdot s^{-1}$	2059 ± 46

Table II. *Effect of White, Red, and Blue Light on the Recovery of the ALA-Synthesizing Capacity*

Twenty h greened seedlings were dark treated as indicated in Table I. Chloroplasts were isolated either at the end of the dark treatment (dark control) or after a 50 min exposure to white, red, or blue light. Isolated chloroplasts were incubated and ALA synthesis was measured. A and B represent two separate experiments. For experimental details, see Table I and "Materials and Methods."

In Vivo Illumination	ALA-Synthesizing Capactiy
	pmol·mg protein^{-1}
A	
Dark control	796 ± 30
White, 6.0 $\mu E \cdot m^{-2} \cdot s^{-1}$	1884 ± 28
Red, 2.7 $\mu E \cdot m^{-2} \cdot s^{-1}$	1996 ± 58
Blue, 3.9 $\mu E \cdot m^{-2} \cdot s^{-1}$	912 ± 28
B	
Dark control	808 ± 20
White, 2.0 $\mu E \cdot m^{-2} \cdot s^{-1}$	1574 ± 28
Red, 0.9 $\mu E \cdot m^{-2} \cdot s^{-1}$	1596 ± 18
Blue, 1.2 $\mu E \cdot m^{-2} \cdot s^{-1}$	899 ± 18

Table III. *Effect of White, Red, and Blue Light on the Reduction of Pchlide and the Recovery of ALA-Synthesizing Capacity*

Two h dark-treated tissue was exposed for 50 min to either white, red, or blue light. Chloroplasts were isolated either at the end of the dark treatment (dark control) or after the 50 min illumination. ALA-synthesizing capacity of the isolated chloroplasts was determined. Pchlide in the isolated chloroplasts was measured before the incubation. For experimental details, see Table I and "Materials and Methods."

In Vivo Illumination	Protochlorophyllide	ALA-Synthesizing Capacity
	pmol·mg protein^{-1}	
Dark control	198 ± 3	743 ± 14
White, 6 $\mu E \cdot m^{-2} \cdot s^{-1}$	95 ± 2	1875 ± 44
Red, 2.7 $\mu E \cdot m^{-2} \cdot s^{-1}$	103 ± 1	1907 ± 39
Blue, 3.9 $\mu E \cdot m^{-2} \cdot s^{-1}$	98 ± 2	878 ± 23

$m^{-2} \cdot s^{-1}$) was essentially ineffective in bringing about restoration of the ALA-synthesizing capacity in dark treated tissue (Tables II and III). Lowering the photon flux density of the three colors to one-third did not change their relative effectiveness (Table II). At the lower intensities of white and red light (2.0 and 0.9 $\mu E \cdot m^{-2} \cdot s^{-1}$, respectively), the extent of restoration of the ALA-synthesizing capacity was still high.

Endogenous Pchlide in the chloroplasts, which were isolated from seedlings at the end of the dark treatment or after the reillumination with white, red, or blue light, was determined before the assay for the ALA-synthesizing capacity (Table III). It can be seen that white (6 $\mu E \cdot m^{-2} \cdot s^{-1}$), red (2.7 $\mu E \cdot m^{-2} \cdot s^{-1}$), and blue light (3.9 $\mu E \cdot m^{-2} \cdot s^{-1}$) all decreased the Pchlide of the dark-treated tissue to approximately the same extent. However, blue light, unlike white or red light, did not restore the ALA-synthesizing capacity (Table III).

Red light applied intermittently in five cycles consisting of 2-min pulses of red light, separated by 10-min dark periods, was nearly as effective as 60 min of continuous red illumination in bringing about restoration of the ALA-synthesizing capacity in dark treated tissue (Table IV), although the total red dose in the intermittent exposure was only one-sixth of that in the continuous exposure. However, if the same dose was given all at once and followed by 50 min darkness, the ALA-synthesizing capacity was not much greater than in the dark control (Table IV), in agreement with the decay of the ALA-forming capacity in the dark previously reported (ref. 12, Fig. 4).

The effect of intermittent red to bring about the restoration of the ALA-synthesizing capacity was almost completely abolished by a far-red pulse following immediately after each red pulse (Table V). The far-red pulses in themselves were not effective in enhancing the ALA-synthesizing capacity (Table V, A and C). Red far-red reversibility and the ineffectiveness of far-red light by itself to induce the response were seen whether the ALA-synthesizing capacity of the isolated chloroplasts was assayed in the light (Table V, A and B) or in the dark with exogenous cofactors (Table V, C). In an intermittent series of red and far red pulses, the effect on the ALA-synthesizing capacity depended on the sequential order of the two pulses (Table V, B). Continuous blue light following a red pulse did not enhance the effect of the red pulse (Table VI).

Table IV. *Effect of Intermittent Red Light on the Recovery of ALA-Synthesizing Capacity*

Dark-treated tissue was subjected to various illumination regimes with red light (2.7 $\mu E \cdot m^{-2} \cdot s^{-1}$). Chloroplasts were isolated either at the end of a 2 h dark treatment (dark control), or after 1 h of dark followed by the designated illumination. ALA-synthesizing capacity of the isolated chloroplasts was determined. For experimental details, see Table I and "Materials and Methods."

In Vivo Illumination	ALA-Synthesizing Capacity
	pmol·mg protein^{-1}
Dark control	750 ± 16
60 min red	1844 ± 38
10 min red + 50 min dark	935 ± 26
(2 min red + 10 min dark) repeated five times	1666 ± 36

Table V. *Inhibition of the Red Light Stimulation by Far-Red Light*

Dark-treated tissue was subjected to various illumination regimes with red and far-red light. Chloroplasts were isolated either at the end of a 2 h dark treatment (dark control), or after 1 h of dark followed by the designated illumination. ALA-synthesizing capacity of the isolated chloroplasts was determined using two different *in organello* assays (11): A and B (photosynthetic assay), 20 min incubation in the light without added ATP or [H]; C (dark assay), 45 min incubation in the dark with added ATP and [H]. For experimental details, see Table I and "Materials and Methods."

In Vivo Illumination	ALA-Synthesizing Capacity
	pmol·mg protein^{-1}
A (photosynthetic assay)	
Dark control	620 ± 0
(2 min red + 10 min dark) repeated 5 times + 2 min red	1663 ± 51
(3 min far-red + 9 min dark) repeated 5 times + 3 min far-red	609 ± 15
(2 min red + 3 min far-red + 7 min dark) repeated 5 times + 2 min red + 3 min far-red	703 ± 20
B (photosynthetic assay)	
Dark control	697 ± 23
(2 min red + 3 min far-red + 7 min dark) repeated 5 times + 2 min red + 3 min far-red	768 ± 27
(3 min far-red + 2 min red + 7 min dark) repeated 5 times + 3 min far-red + 2 min red	1801 ± 45
C (dark assay)	
Dark control	1107 ± 30
(2 min red + 10 min dark) repeated 5 times + 2 min red	2935 ± 28
(3 min far-red + 9 min dark) repeated 5 times + 3 min far-red	1199 ± 23
(2 min red + 3 min far-red + 7 min dark) repeated 5 times + 2 min red + 3 min far-red	1290 ± 31

Cycloheximide (100 μg/mL) applied to cotyledons in the middle of the dark treatment prevented the recovery of the ALA-synthesizing capacity upon reillumination of the tissue with white light (Table VII, A). If CHX was applied to cucumber cotyledons after 1 h of dark treatment and the cotyledons were further incubated in the dark for 1 additional h prior to chloroplast isolation, the protein synthesis inhibitor had no effect on the level of the ALA-synthesizing capacity in the isolated chloroplasts (Table VII, B). Therefore, we see that CHX, applied to dark treated tissue, requires light to exert its effect on ALA synthesis. Moreover, CHX applied directly to isolated chloroplasts (in the same assay that was used in Table VII to measure the effect of CHX applied *in vivo* on the ALA-synthesizing capacity of the isolated chloroplasts) had no effect on light-supported ALA synthesis (data not shown). Likewise, CHX applied to isolated chloroplasts did not affect the synthesis of Pchlide or ALA in the dark with exogenous cofactors (Table VIII) and therefore had no effect on 'ALA over production,' in contrast to what was found by Gough in a study using intact barley shoots (7).

Table VI. *Lack of Synergism Between Blue and Red Light on the Recovery of the ALA-Synthesizing Capacity*

Dark-treated tissue was subjected to various illumination regimes with red and blue light. Chloroplasts were isolated either at the end of a 2 h dark treatment (dark control) or after 1 h of dark followed by the designated illumination. Red light (3.0 $\mu E \cdot m^{-2} \cdot s^{-1}$) and blue light (3.9 $\mu E \cdot m^{-2} \cdot s^{-1}$) were provided as described. For experimental details, see Table I and "Materials and Methods." A and B represent two separate experiments.

In Vivo Illumination	ALA-Synthesizing Capacity
	pmol·mg protein^{-1}
A	
Dark control	777 ± 23
(2 min red + 10 min dark) repeated 5 times	1598 ± 44
2 min red + 58 min blue	871 ± 34
60 min blue	829 ± 7
B	
Dark control	750 ± 16
2 min red + 58 min dark	781 ± 26
2 min red + 58 min blue	805 ± 26

Table VII. *Cycloheximide Applied in Vivo Prevents the Recovery of the ALA-Synthesizing Capacity*

Twenty h greened seedlings were dark treated for 1 h. The cotyledons were excised and vacuum-infiltrated with and without CHX (100 μg/mL) as described in "Materials and Methods." A: Samples of vacuum-infiltrated cotyledons with and without CHX were further exposed to white light (60 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 1 h. Chloroplasts were isolated from three tissues: dark control (2 h dark, no CHX); light exposed (2 h in dark, 1 h in light) with CHX; light exposed without CHX. B: After vacuum infiltration, the cotyledons were incubated for 1 h in the dark without subsequent light treatment. Chloroplasts were isolated from three tissues: the initial tissue before dark treatment; dark-treated tissue infiltrated without CHX; dark-treated tissue infiltrated with CHX. The isolated chloroplasts were assayed for their ability to synthesize ALA in the light for 20 min. For experimental details, see Table I and "Materials and Methods."

In Vivo Illumination	ALA-Synthesizing Capacity
	pmol·mg protein^{-1}
A	
Dark control	828 ± 15
1 h light without CHX	1948 ± 48
1 h light with CHX	785 ± 22
B	
Light control	2331 ± 46
2 h dark without CHX	763 ± 27
1 h dark without CHX + 1 h dark with CHX	744 ± 6

DISCUSSION

About a threefold increase in the ALA-synthesizing capacity assayed *in organello* occurred when dark-treated cucumber seedlings were exposed to white light prior to chloroplast isolation (12). Our experimental findings reported here suggest that this restoration of ALA-synthesizing activity by light is a low fluence phytochrome response (15). This response follows upon illumination with white or red light, but not blue light

Table VIII. *Cycloheximide Applied to Isolated Chloroplasts Has No Effect on Pchlide or ALA Synthesis in the Dark with Exogenous Cofactors*

Chloroplasts were isolated from 20 h greened seedlings and incubated in the dark for 1 h with 6 mM Glu, 3 mM ATP, 4 mM [H], 1 mM SAM, with or without 10 mM LVA and with or without 100 μg/mL CHX. Both Pchlide and ALA were determined for each treatment and expressed as ALA-equivalents.

LVA	CHX	ALA-Equivalents		
		ALA	Pchlide	Total
		pmol·mg protein$^{-1} \cdot h^{-1}$		
−	−	457	1314	1771
−	+	470	1337	1801
+	−	4166	103	4269
+	+	4054	110	4164

(Tables II, III, and VI). Illumination with white, red, or blue light of similar photon flux density gave approximately the same steady state levels of endogenous Pchlide, which were measured in the isolated chloroplasts before incubation (Table III). However, the ability to synthesize ALA was high in the chloroplasts isolated from white or red light-exposed tissue, and low in the chloroplasts from blue light-exposed tissue. This observation rules out the possibility that the difference between red and blue light in our case has to do with different degrees of effectiveness in the phototransformation of Pchlide and strengthens the argument presented elsewhere (12) that the ALA-synthesizing ability in cucumber plastids is not regulated by a Pchlide feedback system. Blue light following a red light pulse did not increase the ALA-synthesizing capacity over red-light alone (Table VI), indicating that there is no synergism between lights of these two spectral regions.

Complete dose response curves were not generated because of the limitations in the apparatus available to us; however, our data (Tables I and II) show that the system is quite responsive to white and red light of moderate intensity. Fifty min of continuous illumination with red light for a total fluence of 0.27 $\mu E/cm^2$ (Table IIB), and five 2-min pulses of red light evenly distributed over a 60 min period, for a total fluence of 0.16 $\mu E/cm^2$ (Table IV), gave near saturating responses.

Table IV also underscores the transient nature of the red light response. The samples on the third and fourth lines received identical doses of red light, but the dose was much more effective when distributed over five 2-min pulses (line 4) than when given during an initial 10 min followed by 50 min of dark (line 3). The transient nature of this red light response confirms the dark decay of the ALA-synthesizing capacity reported in the companion paper (12).

Far red light was not effective in bringing about the enhancement of ALA-synthesizing activity (Table V, A and C). This finding discredits the importance of a very low fluence component in the phytochrome regulation of this response (15). Moreover, far-red illumination, immediately following the red light pulse, abolished the positive response due to red light (Table V, A and C). Repetitive series of red and far-red pulses were effective in restoring ALA-synthesizing capacity if red followed far-red, and ineffective if far-red followed red

(Table V, B). Far-red inhibition of a red-induced response is, unquestionably, the touchstone of a phytochrome-regulated process.

Cycloheximide totally inhibited the recovery of the ALA-synthesizing capacity upon exposure of the dark treated tissue to white light (Table VII, A). Control experiments showed that this inhibitor had absolutely no effect when applied directly to the ALA synthesis assay in isolated chloroplasts. When applied *in vivo*, CHX had no effect unless the tissue was exposed to light (Table VII, B). Therefore, we can conclude that this phytochrome-mediated light response involves translation on cytoplasmic (80 S) ribosomes. Thus, in less than 1 h, this phytochrome response, which involves translation on cytoplasmic ribosomes, and possibly also nuclear transcription (20), exerts a profound influence on an enzyme system located in the chloroplast stroma (8). This means that we are dealing with a fairly rapid photoresponse considering the complexity of the cellular events. To the best of our knowledge, our experimental findings with CHX (Tables VII and VIII) have separated for the first time the effect of light on ALA synthesis from ALA overproduction (7).

When greening barley seedlings were returned to darkness, there was no decrease in the activity of the solubilized ALA-synthesizing enzymes compared with the controls that were kept in the light (13). This disparity between the cucumber and barley systems has not yet been investigated. It could be due to any one of several causes: (a) taxonomic differences (monocot *versus* dicot), (b) differences in the type of tissues (true leaves *versus* epigeous cotyledons), (c) difference in developmental stage of the two tissues, (d) differences in the test materials used to assay for ALA-synthesizing activity (soluble enzymes *versus* intact isolated chloroplasts).

All these factors are potentially able to account for the differences observed between the cucumber and barley systems.

Phytochrome regulation of greening is not new. However, until now it has been shown only on relatively early events in the greening time sequence of dark grown tissues. In this study, we have shown that a phytochrome system regulates the formation of ALA, the first committed metabolite in the synthesis of Chl and other tetrapyrroles, in a tissue that has already accumulated 45% of its final Chl content on a fresh weight basis (12), and is presumably in the terminal stages of greening (4, 14).

Needless to say, phytochrome may not be the only mechanism for the regulation of ALA synthesis in cucumber chloroplasts, since, for one thing, there is about one-third of the ALA-synthesizing capacity, which does not appear to be controlled by phytochrome.

In a context of speculation one should consider that Pchlide feedback inhibition and phytochrome regulation could be regarded as two alternative ways to accomplish the same goal, namely, as two ways of regulating ALA formation depending on whether or not light is available to complete the processes of Chl biosynthesis and membrane biogenesis. It seems very probable that Pchlide feedback inhibition of ALA synthesis would take place exclusively in the chloroplast. On the other hand, phytochrome regulation would also require cytoplasmic and probably nuclear events (20).

In *Euglena* the chloroplasts do not seem to be as closely integrated into the life of the whole cell as they are in higher plants. This is shown, among other things, by the fact that aplastidic *Euglena* mutants are perfectly viable provided they are grown in the presence of suitable organic nutrients. In *Euglena*, Chl synthesis is regulated by Pchlide feedback at the level of ALA formation (9, 19). At the opposite extreme, in cucumber cotyledons, we have found no evidence for Pchlide feedback regulation (12), but only for phytochrome regulation. For other tissues, particularly greening barley shoots, evidence has been found that would support both mechanisms of light regulation (13, 14). The significance of these differences for the ontogeny and evolution of the green plant cell should not be overlooked.

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