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Control of Thylakoid Growth in *Phaseolus vulgaris*

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ABSTRACT

An attempt was made to answer whether the extent of thylakoid growth in *Phaseolus vulgaris* is controlled by a feedback inhibition mechanism, operating after insertion of all of the necessary components of the mature thylakoid, in the right amounts and ratio, or by parameters independent of the developmental stage of the membrane. This was done by following the growth of thylakoids, as monitored by the rate of chlorophyll accumulation and the rate of thylakoid protein synthesis, in etiolated plants exposed either directly to continuous light (transformation of prolamellar body to mature thylakoid) or first to periodic light and then to continuous light (transformation of prolamellar body to primary thylakoids and then to mature thylakoids). It was found that prolonged etiolation has no effect on the rate of thylakoid synthesis in continuous light. However, prolonged preexposure to periodic light diminishes drastically the rate of new thylakoid synthesis in continuous light. Since the thylakoids formed in the latter case are far from being complete, it seems that thylakoid growth can stop long before all of the necessary components are incorporated. Parameters independent of the developmental stage and composition of the membrane, therefore, seem to control membrane growth.

One of the most characteristic changes occurring during the light-controlled differentiation of the etioplast to chloroplast is the transformation of the prolamellar body to the functional stroma and grana thylakoids. This transformation and growth of the photosynthetic membrane occur by a multistep growth process of preexisting membranes (9, 13, 15). Both structural and functional components have to be synthesized, incorporated, assembled, and integrated into functional units during this process. It is not known yet, however, how and when all of these components are integrated, what controls their synthesis and integration, or what controls the extent of the thylakoid growth. Of the thylakoid components—pigments, lipids, and proteins—the site of synthesis of the thylakoid polypeptides has been more extensively studied. It is generally accepted from results with protein synthesis inhibitors that the polypeptides of the thylakoid are of both nuclear transcription-cytoplasmic or chloroplast translation and of chloroplast transcription-chloroplast translation (14, 17–20). In addition, results of experiments in which lipid and Chl *a* incorporation were studied in growing thylakoids (11, 16), have shown that the newly synthesized molecules are inserted throughout the expanding thylakoid, and this precludes the possibility that the thylakoid growth occurs on specific growing sites.

No answer can be as yet offered to the question concerned with the control of the thylakoid growth. Is this growth controlled by the developmental stage of the membrane itself, *i.e.* by some kind of a feedback inhibition mechanism, operating after insertion of all of the necessary components of the mature thylakoid, or is it controlled by parameters independent of the stage of growth of the membrane, like, for example, parameters regulating membrane protein synthesis? To explore this question one should study the way by which thylakoids with minimal composition are transformed to the mature stage. This can be done if etiolated plants are exposed first to periodic light and then transferred to continuous light. Exposure of etiolated leaves to periodic light transforms the etioplasts to protochloroplasts, an intermediate stage of chloroplast development. The protochloroplasts are agranal, and their primary thylakoids have reduced amounts of Chl *a*, they are devoid of Chl *b*, and deficient in the Chl-protein complex II and the 25 to 30 kdalton polypeptides (4, 8–12) believed to be derived from this complex (7). However, they are photosynthetically competent, with high PSI and PSII activity and CO_2 fixation rate (1, 3, 5). In addition, their PSII units are small in size (1). The primary thylakoids, therefore, even though in a quite organized and functional state, are far from being complete, as judged by the composition of the mature thylakoid to which they are transformed after transfer of the periodic light plants to CL.[1] Indeed, the mature thylakoids which develop from the primary thylakoids contain Chl *b*, more Chl *a*, they become gradually enriched in the Chl-protein complex II and the 25 to 30 kdalton polypeptides, they have PSII units of larger size and form grana stacks (1, 8–12).

Our working hypothesis was the following. If the developmental stage of the membrane controls its growth, then the primary thylakoid would be transformed to the mature stage irrespective of the time of exposure to periodic light, as long as it remains incomplete. On the other hand, if the membrane growth is controlled by other parameters, we would expect the duration of preexposure to periodic light to affect further development of the membrane. Hitherto, this transformation of the primary thylakoid to the mature thylakoid stage was studied in leaves transferred to CL after preexposure to periodic light for a limited period of time. The complete transformation under these conditions was always found to take place.

We followed the rate of Chl and thylakoid protein synthesis in etiolated plants exposed first to periodic light and then transferred to CL, and compared the rates found with those of plants, of similar age, exposed directly to CL. We found that prolonged preexposure to periodic light diminished the rate of new thylakoid synthesis after transfer of the plants to CL. On the contrary, the rate of thylakoid synthesis was not diminished in etiolated plants of the same age, exposed directly to CL. These results suggest that membrane growth can stop long before all of the thylakoid components are incorporated, and that parameters regulating membrane protein synthesis probably control the thylakoid membrane growth.

MATERIALS AND METHODS

Etiolated leaves (*Phaseolus vulgaris*, var. red. kidney) were used for the study.

The plants, grown for several days in the dark (Sherer model L Phytotron) at 24 C and 80% humidity, were exposed either to CL (fluorescent and incandescent lamps, 2,000 lux at leaf level) at 24 C, or to periodic illumination (2 min of light alternating with 98 min of dark) (8) at 24 C and 90% humidity. At various times,

[1] Abbreviations: LDC: light-dark cycles; CL: continuous light.

plants were transferred from periodic illumination to CL, the primary leaves were harvested, and their Chl was determined. The Chl was exhaustively extracted as previously described (8) and determined according to Mackinney (22).

The plastids were isolated from 5 g of leaves by homogenization with 50 ml of 0.3 M sucrose-0.05 M phosphate-0.01 M KCl (pH 7.2) buffer, in an Omni-Mixer for 15 sec at 35% followed by 10 sec at 58% of the line voltage. The homogenate was filtered through six layers of gauze and centrifuged at 500*g* for 2 min. The chloroplasts (plastids of leaves exposed to CL) were collected by centrifuging the supernatant for 10 min at 1,500*g*; the protochloroplasts (plastids of leaves exposed to periodic light) were collected by centrifuging the supernatant for 10 min at 5,000*g*.

The pellets were successively washed with 0.05 M Tricine-NaOH (pH 7.2), ice-cold distilled H_2O, and finally 90% acetone for five times. The thylakoid protein was completely dissolved by boiling in 0.24 N NaOH for 30 min. Protein was determined according to Lowry *et al.* (21). Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter.

RESULTS

Figure 1 shows the accumulation of Chl in etiolated bean leaves exposed either directly to CL or following preexposure to periodic light. The amount of Chl accumulating in 6-day etiolated leaves exposed to periodic light is only about 10% of that formed in leaves exposed to CL; only Chl *a* is formed in this case (8). Transfer of these leaves to CL induces the synthesis of more Chl *a* and synthesis of Chl *b*. As the time of preexposure to periodic light increases, the amount of Chl accumulating after transfer to continuous light is gradually reduced: in plants preexposed up to about 40 LDC, the maximum amount of Chl formed after transfer to CL is almost equal to that of plants exposed directly to CL (on a g fresh wt basis); on the contrary, in plants preexposed to more than about 50 LDC, the maximum amount of Chl formed is drastically reduced. This does not seem to be an age effect, since plants of the same age exposed directly to CL form large amounts of Chl (compare the 11- and 13-day plants with those preexposed to 57, 86, or 114 LDC). The noticeable trend in Chl accumulation to decrease at the plateau to a more or less constant level seems to result from the expansion and growth of the leaf itself. Early after exposure to CL, the growth of the leaf is limited, but the photoinduced formation of chloroplasts and their thylakoids is enhanced; as a result, the Chl/g fresh wt increases. Later on, however, even though the growth of the leaf continues, chloroplast and thylakoid synthesis seems to cease. The constant level of Chl is finally reached when the growth of the leaf comes to a plateau. This was checked experimentally, and the results are shown in Figure 2. Apart from this effect, an age effect on both leaf growth and Chl accumulation was also noticed. Older plants have reduced rate of Chl synthesis and leaft growth. This age effect has been also noticed earlier in the regeneration of Pchl(ide), the lag phase in Chl synthesis as well as on the accumulation of Chl in periodic or CL (2, 6). In the present study it was also found that as the time of preexposure to periodic light increases, a noticeable lag in Chl synthesis becomes evident when the periodic light plants are transferred to CL (Fig. 3). No lag phase is noticed in plants preexposed up to 40 LDC, while there is a clear lag of about 2 hr in those preexposed to 40 LDC. The duration of the lag increases as preexposure to LDC increases. Chl synthesis in CL starts after about 4 hr in leaves preexposed to 57 LDC and after 6 hr in those preexposed to 86 LDC (see also Fig. 1). A very long lag phase seems to occur after 114 LDC which may or may not be a real lag, since the net Chl synthesis in this case is also very low. Similarly, the Chl *a* to Chl *b* ratio in the plants transferred to continuous light depends also on preexposure to periodic light (Fig. 4). The ratio drops faster to the normal value of the mature green leaf in plants preexposed to short periodic light treatment. In view of the evidence which correlates grana formation with Chl *b* synthesis, and assuming that a similar situation occurs also under the experimental conditions of this study, these results may show that grana are formed faster in plants transferred to continuous light after short preexposure to periodic light, while synthesis is inhibited

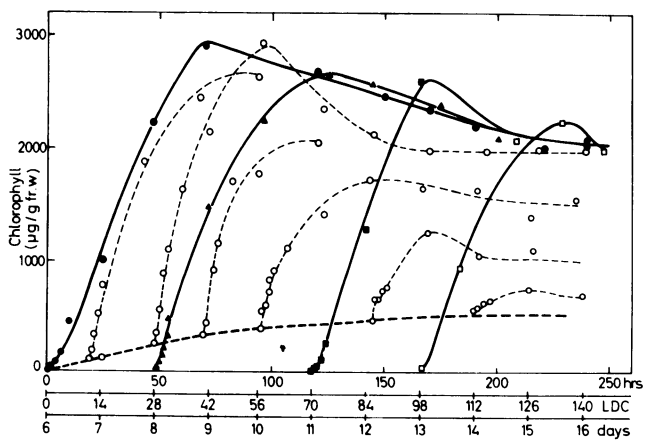

FIG. 1. Synthesis of Chl in etiolated leaves exposed directly to CL, to periodic LDC or to periodic light, and then transferred to CL. (—): direct exposure to CL of 6-day (●——●), 8-day (▲——▲), 11-day (■——■), and 13-day plants (□——□). (---): direct exposure of 6-day etiolated plants to periodic LDC. (---): leaves transferred to CL after preexposure to 11, 28, 42, 57, 87 and 114 cycles (left to right).

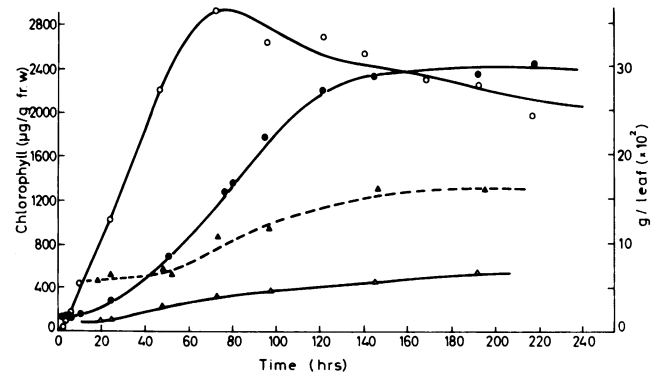

FIG. 2. Comparison of leaf growth (g fr wt/leaf) and Chl accumulation (µg/g fr wt) in 6-day plants exposed to continuous (○——○) or periodic illumination (△——△). Leaf growth: (●——●), CL; (▲——▲), periodic light. Chl synthesis: (○——○), CL; (△——△), periodic light.

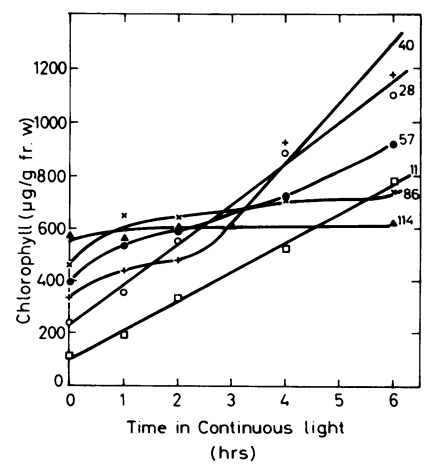

FIG. 3. Effect of time of preexposure to periodic light on the lag phase of Chl synthesis in 6-day etiolated bean plants. Leaves transferred to CL after 11 (□), 28 (○), 40 (+), 57 (●) 86 (×), and 114 (▲) LDC.

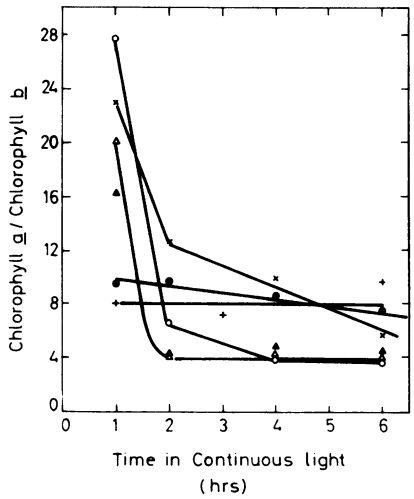

FIG. 4. Effect of time of preexposure to periodic light on Chl *a* to Chl *b* ratio in 6-day etiolated bean plants after transfer to CL. Leaves transferred to CL after 11 (△), 28 (○), 40 (▲), 57 (×), 86 (●), and 114 (+) LDC.

Table I. Effect of prolonged periodic light illumination on the rate of thylakoid chlorophyll and protein synthesis after transfer of the plants to continuous illumination.

C^{14}-Leucine was brushed on 5-day etiolated bean leaves, having one cotyledon removed (25 μCi/2 ml solution, 100 beans) and placed in covered petri dishes prior to exposure to periodic light. The excess leucine was washed off after 14 light-dark cycles. The leaves were replaced in periodic light and then transferred to continuous light.

Illumination conditions	Specific Radioactivity in thylakoid protein	Chl (a+b) / g fr wt	Chl a / Chl b
	cpm/mg		
27 LDC	70,000	85	57
27 LDC+24 hr CL	30,000	1579	3.2
85 LDC	16,000	463	22
85 LDC+24 hr CL	18,000	828	4.7

after extensive preexposure to LDC. This may be due to a block in the synthesis or incorporation of new components, which for some reason occurs as preexposure to periodic light is prolonged.

The effect of prolonged preexposure to periodic light on Chl synthesis seems to reflect a general phenomenon affecting thylakoid formation; this was demonstrated by determining the rate of thylakoid protein synthesis in plants to which [^{14}C]leucine was administered prior to periodic light exposure. Table I shows results of such experiments. The specific radioactivity of the thylakoid protein is reduced to about one-half of the initial value found at 27 LDC, after 24 hr CL. On the contrary, no change in the specific radioactivity of the thylakoid protein is found in plants exposed to 85 LDC or 85 LDC plus 24 hr CL. Under the latter conditions, no appreciable, if any, thylakoid protein synthesis occurs during the 24 hr of CL. The primary thylakoid synthesis in periodic light, however, is not affected by the prolonged exposure, as judged by the rate of Chl synthesis and the specific radioactivity of the thylakoid protein at 27 and 85 LDC.

DISCUSSION

This study showed that in plants transferred to CL after prolonged preexposure to periodic illumination a progressive inhibition occurs in both Chl and thylakoid protein synthesis. This points to a control of membrane growth independent of the developmental stage of the thylakoid and hence of membrane composition, since, even though the primary thylakoid formed in periodic light is still incomplete, it can not be transformed to the mature stage after transfer to CL.

What the parameters are which affect thylakoid growth in CL, and what the reason is for this block are not yet understood and remain unanswered. One of the possible explanations that may be offered is that after prolonged periodic light exposure the primary thylakoid acquires a certain organization which does not permit the incorporation of all of the components of the mature thylakoid. A second possibility could be that certain Chl-bearing polypeptides cannot be formed due to a block in protein synthesis, affecting directly the chloroplastic or cytoplasmic ribosomes, both of which cooperate in the synthesis of the thylakoid proteins (14, 17–20). Finally, one could visualize a direct effect on Chl synthesis itself, which in turn affects the thylakoid protein synthesis. Such a control has been proposed to regulate the greening of *Chlamydomonas reinhardii*, where the photoreduction of Pchl(ide) to Chl(ide) is believed to control the synthesis of the L protein, a membrane protein of cytoplasmic origin (15). Since both Chl and thylakoid protein synthesis in CL seem to be similarly affected by prolonged periodic light pretreatment, the results can not distinguish between these possibilities. Our results, however, have shown that Chl *b* synthesis after transfer to continuous light is drastically affected by prolonged periodic light preexposure (Fig. 4), suggesting that some components of the mature membrane may be more specifically implicated.

In plants transferred to continuous light after short periodic light treatment, the differentiation of the lamellar system to grana and stroma lamellae proceeds normally. This differentiation is accompanied by drastic changes in the composition of the membrane. In periodic light, no Chl *b* or the Chl *a* + *b*–protein complex II can be detected, while the Chl *a*-rich complex I and the 60-kdalton range polypeptides predominate (8–12). After transfer to continuous light, Chl *b* synthesis is triggered and the complex II and the 25- to 30-kdalton polypeptides are formed in excess (8–12). This latter complex which seems to be coded by nuclear DNA (20) is a major component of grana stacks and has been implicated in the stacking of thylakoids during grana formation (9, 12). Since Chl *b* is also a major component of grana stacks under normal greening, and its formation parallels the formation of complex II—and assuming that this is true in our experimental conditions as well—our results suggest that the block in further thylakoid growth may be centered on the differentiation of the primary thylakoid to grana and stroma lamellae. Experiments are underway to explore this point further.

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