Journal of Cellular Biochemistry 23:181–189 (1983)
Biosynthesis of the Photosynthetic Apparatus:
Molecular Biology, Development and Regulation 109–117

The Light-Dependent Control of Chloroplast Development in Barley (Hordeum vulgare L)

Klaus Apel, Ingrid Gollmer, and Alfred Batschauer

Botanisches Institut der Christian-Albrechts Universität Kiel, 2300 Kiel, Federal Republic of Germany

The light-induced greening of etiolated barley plants is used as a model to study the light-dependent control of plastid development. Upon illumination a rapid transformation of etioplasts to chloroplasts is induced. The effect of illumination does not only include the light-dependent chlorophyll synthesis but also the appearance or decline of specific proteins within the plastid membrane fractions. So far two of these proteins have been studied in detail. The light-harvesting chlorophyll *a/b* protein (LHCP) is one of the major protein constituents of the thylakoid membrane of chloroplasts. However, this protein is not detectable among the membrane polypeptides of etioplasts. Illumination of dark-grown barley plants induces a massive insertion of the LHCP. The appearance of the protein is controlled by the cooperation of at least two distinct photoreceptors: protochlorophyllide and phytochrome. In dark-grown barley plants not only the LHCP but also its mRNA is not detectable. The light-dependent appearance of mRNA activity for the LHCP is under the control of phytochrome (P_{fr}). Even though the appearance of mRNA activity is induced via P_{fr} by a single red light pulse, the assembly of the complete LHCP takes place only under continuous illumination, which allows chlorophyll synthesis. The second protein analyzed so far is the NADPH-protochlorophyllide-oxidoreductase. This enzyme catalyzes the light-dependent reduction of protochlorophyllide to chlorophyllide and thus controls one of the first detectable light-dependent reactions during the greening period. It is generally assumed that this enzyme is responsible for the overall chlorophyll synthesis and accumulation during the greening period. In contrast to this hypothesis, we found a rapid decline of the enzyme during illumination. In addition to the decrease of the enzyme protein, the translatable mRNA coding for the enzyme also declines rapidly under the influence of light. Also this effect is mediated by phytochrome. Using cloned cDNA as hybridization probes we have demonstrated that the light-induced changes of the two translatable mRNAs for the NADPH-protochlorophyllide oxidoreductase and the LHCP are both paralleled by corresponding changes in the steady-state concentration of the mRNA sequences. Thus, it seems likely that one major point of control at which phytochrome regulates the development of chloroplasts is the expression of genes at the level of transcription.

Key words: barley, chloroplast development, phytochrome, NADPH-protochlorophyllide oxidoreductase

Received May 26, 1983; revised and accepted September 9, 1983.

Upon illumination of dark-grown higher plants a rapid transformation of etioplasts into photosynthetically competent chloroplasts is induced [1].

The dramatic changes during the light-induced transformation of etioplasts to chloroplasts that occur within a relatively short period of time have made this system an attractive model for studies of the molecular mechanisms that govern light-dependent processes during the development of higher plants. One of the most intriguing problems is the identification of the photoreceptors that regulate the light-dependent changes within the etioplast. The reaction that has been most frequently implicated as the primary target for the action of light on plastid development in higher plants is the photoreduction of protochlorophyllide to chlorophyllide, protochlorophyllide being the photoreceptor of its own reduction [2].

Since the work of Withrow et al [3], however, it has become evident that besides protochlorophyllide a second photoreceptor, the phytochrome system, plays a crucial role during the etioplast transformation in higher plants.

The occurrence of at least two different photoreceptors, both of which are operating simultaneously during the light-induced chloroplast formation, leads to the question of how and to what extent the various developmental processes within the plastid are controlled by each of them. Such an analysis is complicated by the fact that at least two different genetic systems are involved in the control of plastid development [4,6]. Plastids possess their own DNA and protein synthesis system. However, the majority of plastid proteins is coded for by nuclear DNA, synthesized outside the plastid on cytoplasmic ribosomes, and posttranslationally transported into the plastid [7].

In the present work the light-dependent transformation of etioplasts to chloroplasts in barley has been used as a model system to study some of the control mechanisms by which light regulates the intimate cooperation of the two photoreceptors.

MATERIALS AND METHODS

Growth of Plants

Barley (Hordeum vulgare L, cv Carina) was grown in darkness for 5 days as described earlier [8]. In some experiments dark-grown plants were exposed to continuous white light for 16 hr. The conditions for the red light and far-red light treatments of dark-grown barley plants were identical to those used previously [9,10].

In Vitro Translation of Poly(A)-Containing RNA

The isolation of poly(A)-containing RNA and its translation in a wheat-germ, cell-free, protein-synthesizing system; the immunoprecipitation of specific products in vitro; and the electrophoretic separation and detection of products labeled in vitro were done as described elsewhere [8].

The Isolation and Characterization of cDNA Clones

Polysomal poly(A)-containing RNA isolated from illuminated and dark-grown barley plants was used for the synthesis of double-stranded cDNA. The methods for cloning the cDNA in the plasmid pBR322 and for the subsequent selection of those clones that contained cDNA sequences encoding the apoprotein of the light-harvesting

chlorophyll *a/b* protein and the NADPH-protochlorophyllide oxidoreductase have been described elsewhere [11].

RESULTS AND DISCUSSION

The light-dependent assembly of the light-harvesting chlorophyll *a/b* protein in barley demonstrates the close interdependence of the two photoreceptors, protochlorophyllide and phytochrome, and the interaction of the two genetic systems in the plastids and the nucleus, respectively. The two constituents of the complex, chlorophyll and the apoprotein, are synthesized within different compartments of the cell and are both under the control of phytochrome. Light-dependent reduction of protochlorophyllide occurs within the plastid. The accumulation of translatable mRNA for the apoprotein of the light-harvesting chlorophyll *a/b* protein is triggered by phytochrome and leads to the massive synthesis of the apoprotein on 80S ribosomes within the cytosol. The assembly of the complete chlorophyll-protein depends on a close coordination of both biosynthetic pathways by light [8,9].

According to present models the photoreduction of protochlorophyllide as well as the transfer of the freshly formed chlorophyllide from its site of synthesis to its ultimate site of function within the thylakoid membrane includes the participation of a photoactive protein complex, the protochlorophyllide holochrome [1,12 -14]. Until recently none of the molecular events underlying these processes have been known. One reason for this ignorance was the lack of information on the protochlorophyllide holochrome. Even though this protein has been known and studied intensively for a number of years [15], it had not been purified to such an extent that its exact structure and subunit composition could be determined. The finding that NADPH can be used as hydrogen donor for the photoreduction of protochlorophyllide in vitro has enabled Griffiths [16,17] to set up an assay for the determination of the protochlorophyllide-reducing enzyme activity in etioplasts. Based on this work it became possible to solubilize the NADPH-protochlorophyllide oxidoreductase and to purify it to apparent homogeneity [18,19]. The enzyme of barley is composed of a polypeptide of an apparent molecular weight of 36,000 [18].

It has been suggested that the protochlorophyllide-reducing enzyme in dark-grown plants is responsible for the overall chlorophyll synthesis during the light-induced greening [15]. Thus, one would expect to find the NADPH-protochlorophyllide oxidoreductase to be present throughout the greening period. However, contrary to these expectations, Mapleston and Griffiths [20] found a rapid light-induced decrease of the enzyme activity. They interpreted their finding as a reversible inactivation of the enzyme by the light-induced alteration of the plastid NADP pool. Recent work by Santel and Apel [21] has confirmed this observation. However, the light-induced decrease of the enzyme activity in barley was accompanied by a rapid decline of the enzyme protein (Fig. 1). There are several mechanisms by which light might affect the enzymes One possibility, which we have studied, is a light-dependent change in the capacity of an etiolated plant to synthesize the enzyme polypeptide.

In a first attempt to characterize the biosynthetic pathway of the enzyme protein, high-temperature-induced deficiency of plastid ribosomes was used as a system for studying the role of the cytosol in the synthesis of the NADPH-protochlorophyllide oxidoreductase [22]. The enzyme was synthesized in high-temperature-grown barley

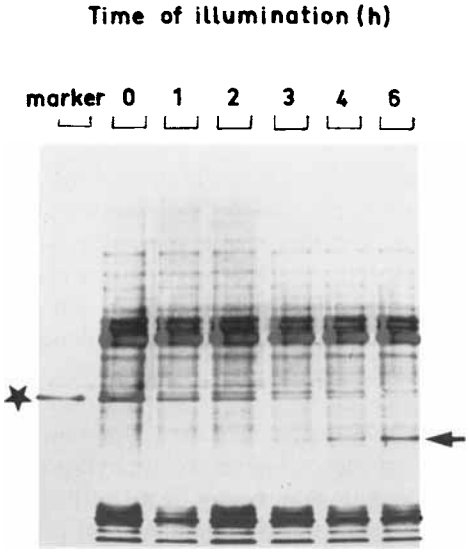

Fig. 1. Light-dependent changes in the polypeptide composition of plastid membranes of barley. The plastid membranes were isolated from dark-grown barley that had been illuminated for different lengths of time. The membrane proteins were solubilized in the presence of sodium dodecyl sulfate and separated electrophoretically. The arrow indicates the position of the light-harvesting chlorophyll *a/b* protein, and the asterix marks the position of the 36.000 M_r polypeptide of the NADPH-protochlorophyllide oxidoreductase.

plants to the same extent as in control plants grown at 25°C [23]. From these results it has been concluded that the NADPH-protochlorophyllide oxidoreductase is synthesized on 80S ribosomes. Similar results have been obtained with other cereal plants [24]. They are consistent with our finding of a polyadenylated mRNA that codes for a high molecular weight precursor of the NADPH-protochlorophyllide oxidoreductase [10]. Higher molecular weight precursors have been described also for other plastid proteins that are synthesized outside the plastid on 80S ribosomes [7]. In darkgrown barley plants the mRNA coding for the NADPH-protochlorophyllide oxidoreductase is one of the most abundant translatable mRNAs. During illumination, the concentration of this mRNA activity rapidly decreases (Fig. 2). This light effect is also controlled by phytochrome as demonstrated by red/far-red reversal experiments [10]. Two conclusions can be drawn from these results. First, it is likely that the light-induced decrease of the mRNA activity contributes to the rapid decline of the enzyme protein during illumination of dark-grown barley plants. Second, the results on the NADPH-protochlorophyllide oxidoreductase together with those on the light-harvesting chlorophyll *a/b* protein demonstrate that one major point of control at which light regulates the transformation of etioplasts to chloroplasts is a phytochrome-mediated change in the level of specific mRNA activities outside the plastids.

One of the intriguing problems which has been brought up by our work is that of the function of the NADPH-protochlorophyllide oxidoreductase during the greening process. Two different proposals are being discussed at the present time. According to Griffiths and co-workers [20], the remaining activity of the NADPH-

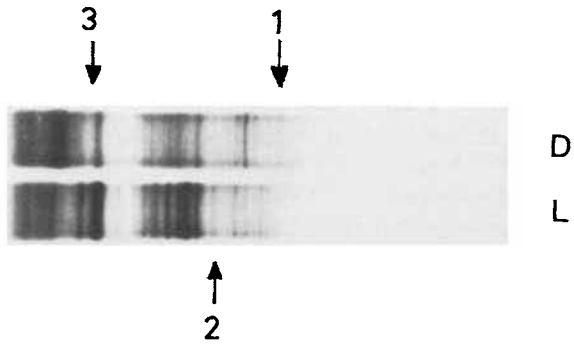

Fig. 2. Light-dependent changes in the pattern of [^{35}S]-labeled products synthesized in vitro in the presence of poly(A$^+$)-RNA fractions, which were isolated from dark-grown (D) or illuminated (L) barley. The arrows from top to bottom mark the position of the NADPH-protochlorophyllide oxidoreductase precursor (1), the light-harvesting chlorophyll *a/b* protein precursor (2), and the "15,000" polypeptide (3).

protochlorophyllide oxidoreductase in illuminated plants should be sufficiently high to account for the overall light-induced chlorophyll synthesis. In contrast to this view,we propose that the enzyme may be functionally important only during the initial phase of greening [21]. At the time of illumination at which chlorophyll accumulation reaches its highest rate, only traces of the enzyme protein are still detectable within the membrane, and the enzyme activity has dropped beyond the limit of detection, which is less than 2% of the activity present in dark-grown plants [21]. Furthermore the light-induced decline of the mRNA encoding the enzyme protein gives further support to the idea that during the later stages of greening enzymes other than the NADPH-protochlorophyllide oxidoreductase may be responsible for the chlorophyll accumulation. Recent results from several laboratories clearly show that the biosynthesis of chlorophyll in higher plants is much more complex than previously assumed [cf. 25].

By the methods used it was not possible to distinguish between a phytochrome-dependent activation of preformed mRNA sequences (eg, by initiation of processing) and a phytochrome-induced change in the amount of specific mRNA sequences. In order to solve this problem we have cloned hybridization probes for the apoprotein of the LHCP and the NADPH-protochlorophyllide oxidoreductase. cDNA clones were constructed by standard methods, using total polysomal poly(A$^+$)-RNA from etiolated and illuminated barley plants to synthesize cDNA by reverse transcriptase [11].

Two different strategies were used for the screening of the bacterial colonies. First, all tetracycline-resistant and ampicillin-sensitive colonies were screened by in situ colony hybridization using [^{32}P]-cDNAs as probes, which had been synthesized from dark-or light-specific polysomal poly(A)-containing RNA. Most colonies hybridized similarly to both probes, but some showed strong preferential hybridization for the light-or dark-specific cDNA probe. These colonies were selected for further analysis. For the next screening step poly(A)-containing RNA from illuminated or dark-grown barley leaves was fractionated by centrifugation through an exponential

sucrose gradient. From the gradients, fractions were obtained that were enriched in mRNA, encoding the apoprotein of the LHCP and the NADPH-protochlorophyllide oxidoreductase. From these RNA fractions [^{32}P]-cDNAs were synthesized, which were used as hybridization probes. Out of the light- or dark-specific cDNA clones that had been selected during the first screening step, several gave strong hybridization signals with the cDNA probes [11] (Fig. 3).

The identity of the various clones was confirmed by hybridization selection of RNA from total poly(A)-containing RNA using the plasmid DNA bound to nitrocellulose. The plasmid DNAs of clones specific for the apoprotein of the LHCP [11] and the NADPH-protochlorophyllide oxidoreductase were used for further experiments.

Dark-grown barley plants were exposed to a 15-sec red light pulse and transferred back to darkness. After different lengths of time following the red light treatment, poly(A)-containing RNA was isolated from the leaves and analyzed in two different ways. First, equal amounts of RNA were translated in a cell-free wheat germ system and the relative concentration of the mRNA activity for the light-harvesting chlorophyll *a/b* protein was determined. Following the red light pulse the relative proportion of the precursor in vitro to the apoprotein of the LHCP increased rapidly during the subsequent dark period and reached its maximum after 7.5 hr. After 10 hr the concentration of the precursor molecule began to decrease (Fig. 4A).

In a second experiment equal amounts of RNA of the same preparations as used in the previous experiment were glyoxylated and separated electrophoretically on an agarose gel. The RNA was transferred to nitrocellulose paper and hybridized to nick-translated [^{32}P]-pHvLF2 DNA, which contains a cDNA insert encoding a constitutive polypeptide of the light-harvesting chlorophyll *a/b* protein [11].

In the dark sample, no radioactivity could be detected. After 2.5 hr. a hybridization band could be seen whose intensity increased rapidly during the next 5 hr (Fig. 4B). These results indicate that not only continuous white light but also a single red light pulse is sufficient to affect drastically the mRNA coding for the apoprotein of the LHCP. It is evident that not only the amount of the activity but also the steady-state concentration of the mRNA sequence increased rapidly during the subsequent dark period. In both experiments, the maximum response was reached after 7.5 hr of dark incubation following a single red light pulse. This dark period of 7.5 hr was chosen in another experiment in which the reversibility of the red light treatment by irradiation with far-red light was tested.

The far-red light reversed substantially the effect of the red light treatment [11]. Only traces of the precursor molecule were detectable in the assay for mRNA activity in which the RNA of the red/far-red light sample had been used as template for protein synthesis. The hybridization of the same RNA sample with the nick-translated [^{32}P]-DNA probe revealed little if any radioactivity even after prolonged exposure.

These results demonstrate that the actual sequence content of the mRNA encoding the apoprotein of the LHCP is under the control of phytochrome. Similar results on the stimulating effect of phytochrome on nuclear-encoded proteins have been obtained also with pea and duck weed [26–28]. Thus, a phytochrome-dependent stimulation of the steady-state concentration of specific RNAs is not confined to barley plants but appears to be of more general importance. Even though at present it cannot be excluded that phytochrome may also affect the stability of these transcripts, these results suggest to us that phytochrome may rapidly affect the transcription of specific genes. Such a suggestion is supported by recent results on the transcription

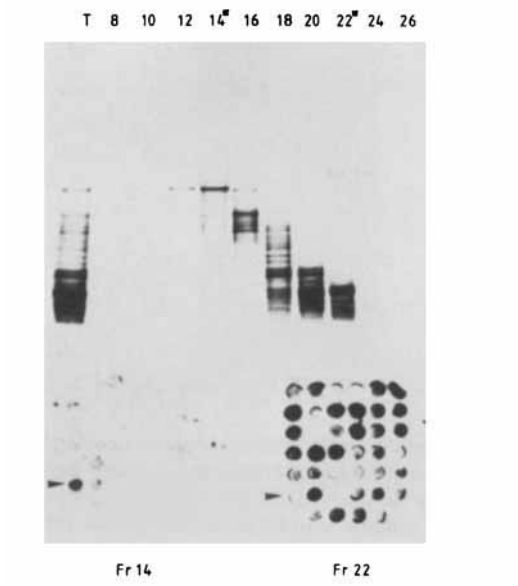

Fig. 3. Colony hybridization screening for recombinant cDNA clones containing sequences coding for the NADPH-protochlorophyllide oxidoreductase and a "15,000" polypeptide. The poly(A)-containing RNA of dark-grown barley plants was separated on an exponential sucrose gradient, and those fractions were pooled which were enriched in poly(A)-containing RNA specific for the NADPH-protochlorophyllide oxidoreductase (FR.14) and the "15,000" polypeptide (Fr.22). After labeling with ^{32}P the two fractions were used as probes to screen the "dark"-specific bacterial colonies.

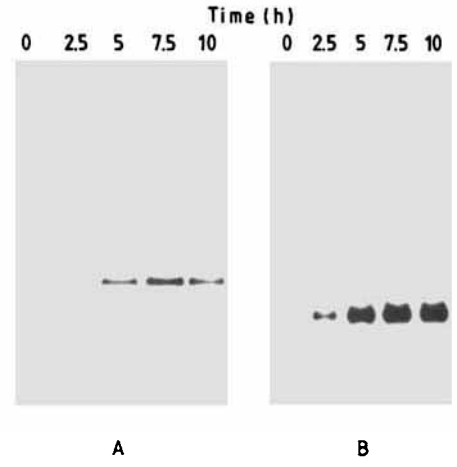

Fig. 4. The red light-induced increase in the mRNA activity (A) and the steady state concentration of transcripts (B) encoding the light-harvesting chlorophyll *a/b* protein (LHCP) of barley. Dark-grown plants were exposed to a single red light pulse and transferred back to darkness. After different lengths of time the polyadenylated RNA was isolated from the leaves. For the determination of mRNA activity, equal amounts of RNA were translated in the wheat germ system and the precursor in vitro to the LHCP was immunoprecipitated, separated electrophoretically, and detected by autoradiography. The concentration of RNA sequences hybridizing with the LHCP-specific cDNA clone pHvLF2 was determined by Northern blot hybridization [30].

Fig. 5. The red-light induced rapid decrease of RNA sequences coding for the NADPH-protochlorophyllide oxidoreductase. RNA samples and experimental conditions were the same as in Figure 4B, except that a cDNA clone specific for the NADPH-protochlorophyllide oxidoreductase (pHvDF1) was used as hybridization probe. The numbers indicate the lengths of time (hr) of dark incubation after the initial red light treatment.

of specific genes in isolated nuclei. In such experiments only newly synthesized transcripts rather than the total content of specific RNA sequences have been determined [28,29].

As shown in our earlier work on the NADPH-protochlorophyllide oxidoreductase,phytochrome does not only mediate an increase in the level of some specific mRNAs but at the same time also exerts an inverse effect on other specific mRNA activities (Fig. 2). The hybridization probe coding for the NADPH-protochlorophyllide oxidoreductase was used in a similar experiment as that described for the light-harvesting chlorophyll *a/b* protein to study this regulation phenomenon. Dark-grown barley plants were exposed to a single red light pulse and returned to darkness. After various lengths of dark incubation, poly(A^+)-RNA was isolated, and the concentration of the RNA sequences hybridizing to the NADPH-protochlorophyllide oxidoreductase-specific probe was determined by Northern blot hybridization on nitrocellulose filters [30]. During the first hour the concentration of the mRNA sequence coding for the NADPH-protochlorophyllide oxidoreductase increased, followed by a rapid decline in concentration during the next 90 min (Fig. 5). Two conclusions may be drawn from this observation. First, the kinetic for the decline of the dark-specific mRNA lends support to the idea that more than one mechanism is involved in the light-induced decrease of the mRNA sequence. Second, our results suggest that in addition to a light-induced termination of transcription, which is likely to occur, the existent RNA sequences might be rapidly degraded following the red light pulse. It is possible that upon illumination the RNA is modified such that it becomes susceptible to preexistent nucleases, or as an alternative, specific nucleases might be activated after the light treatment.

One point of control by which phytochrome regulates the light-dependent development of the chloroplast has been identified. Several plastid proteins, including the light-harvesting chlorophyll *a/b* protein, the NADPH-protochlorophyllide oxidoreductase, and the small subunit of the ribulose-1, 5-bisphosphate carboxylase, all of which are synthesized outside the plastid,are affected by light in the same compart-

ment; phytochrome controls the steady-state level of the mRNA sequences encoding these proteins. It also is possible that the other plastid proteins that are encoded by nuclear DNA and affected by phytochrome are regulated in the same way. The work done so far does not give an ultimate answer to the problem of how phytochrome exerts its control on specific genes. However, the cDNA probes do not only allow the detection of specific transcripts but also the isolation of the corresponding genes and thus should offer a powerful tool for elucidating the molecular basis of phytochrome action during gene expression in the future.

ACKNOWLEDGMENTS

Original research from the author's laboratory referred to in this article was funded by the Deutsche Forschungsgemeinschaft (S.F.B. 46, E 10; Ap 16/4-1, Ap 16/4-2).

REFERENCES

1. Boardman NK, Anderson JM, Goodchild, DJ: Curr Top Bioenerget 8:35, 1978.
2. Koski VM, French CS, Smith FHC; Arch Biochem 31:1, 1951.
3. Withrow RB, Wolff JB, Price L: Plant Physiol 31 (Suppl XIII), 1956, 13–14.
4. Bogorad L: Science 188:891, 1975.
5. Ellis RJ: Annu Rev Plant Physiol 32:111, 1981.
6. Kirk JTO, Tilney-Bassett RAE: "The plastid: Their Chemistry, Structure, Growth and Inheritance." London and SanFrancisco: WH Freeman, 1967.
7. Chua NH, Schmidt GW: J Cell Biol 81:461, 1979.
8. Apel K, Kloppstech K: Eur J Biochem 85:581, 1978.
9. Apel K: Eur J Biochem 97:183, 1979.
10. Apel K: Eur J Biochem 120:89, 1981.
11. Gollmer I, Apel K: Eur J Biochem 133:309, 1983.
12. Bogorad L: In Goodwin TW (ed): "Chemistry and Biochemistry of Plant Pigments." London: Academic Press, 1976, pp 64–148.
13. Bogorad L, Laber L, Gassman M: In Shibata K, Takamiya A, Jagendorf AT, Fuller RC (eds): "Comparative Biochemistry and Biophysics of Photosynthesis." Baltimore: University Park Press, 1968, pp 299–312.
14. Harel E: In Reinhold JB, Harborne JB, Swain T (eds): "Progress in Phytochemistry,' Vol 5 New York: Pergamon Press, 1978, pp 127–180.
15. Boardman NK: In Vernon LP, Seely GR (eds): "The Chlorophylls." New York: Academic Press, 1966, pp 437–479.
16. Griffiths WT: FEBS Lett 46:301, 1974.
17. Griffiths WT: Biochem J 174:681, 1978.
18. Apel K, Santel HJ, Redlinger TE, Falk H: Eur J Biochem 111:251, 1980.
19. Beer NS, Griffiths WT: Biochem J 195:83, 1981.
20. Mapleston RE, Griffiths WT: Biochem J 189:125, 1980.
21. Santel HJ, Apel K: Eur J Biochem 120:95, 1981.
22. Feierabend J: Ber Dtsch Bot Ges 92:553, 1979.
23. Batschauer A, Santel HJ, Apel K: Planta Med (Berl) 154:459, 1982.
24. Griffiths WT, Beer NS: Plant Physiol 70:1014, 1982.
25. Kasemir H: Photochem Photobiol 37:701, 1983.
26. Hartley MR, Jenkins GI, Cuming AC, Bennet J: NATO Adv Study Inst Book of Abstracts, p125, 1982.
27. Everett M, Polans N, Jorgensen RA, Palmer GD, Thomson WF: NATO Adv Study Inst Book of Abstracts, P 64, 1982.
28. Tobin EM, Silverthorne J, Stiekema WJ, Wimpee CF: In Ellis RJ (ed): 'Chloroplast Biogenesis.' Cambridge: Cambridge University Press (in press).
29. Gallagher F, Ellis RJ: EMBO J 1:1493, 1982.
30. Thomas PS: Proc Natl Acad Sci USA 77:5201, 1980.