Plant Cell Physiol. 44(8): 844–855 (2003)

Light and Cytokinin Play a Co-operative Role in MGDG Synthesis in Greening Cucumber Cotyledons

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The current research investigated the regulation of monogalactosyldiacylglycerol (MGDG) biosynthesis, catalyzed by MGDG synthase (MGD) (UDP-galactose:1,2-diacylglycerol 3-β-D-galactosyltransferase; EC 2.4.1.46), during chloroplast development in cucumbers (*Cucumis sativus* L. cv. Aonagajibai). In etiolated seedlings, white light induced a transient increase in MGD mRNA, followed by a subsequent increase in enzyme activity. MGDG, digalactosyldiacylglycerol (DGDG), and linolenic acid (18 : 3) of both MGDG and DGDG accumulated in a light-dependent manner. Early light-dependent induction of MGD protein was also identified in isolated chloroplasts. When cotyledons were detached from seedlings, these light-induced changes diminished. However, when a synthetic cytokinin, benzyladenine, was added to the detached cotyledons, a transient increase in MGD mRNA and a linear increase in the enzyme activity were induced even in the dark. Galactolipids subsequently accumulated to some extent and 18 : 3 content also increased. MGDG fully accumulated in detached cotyledons with co-treatment of light and a cytokinin. Red light (>600 nm) and far-red light (>700 nm) both induced an increase in MGD mRNA and enzyme activity but far-red light did not induce an accumulation of MGDG. These results suggest that (1) galactolipid biosynthesis is regulated by the cooperation of light and a cytokinin; (2) the accumulation of MGDG requires cytokinin in addition to light; (3) a red light (600–700 nm) dependent factor is necessary for the maximal galactolipid accumulation in addition to increase in MGD transcript and activity.

Keywords: Chloroplast development — Cytokinin — Desaturase — Galactolipids — Light response — MGDG synthase.

Abbreviations: BA, benzyladenine; DG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DTT, dithiothreitol; *lh*, long hypocotyl; MGD, UDP-galactose:1,2-diacylglycerol 3-β-D-galactosyltransferase; *MGD*, MGDG synthase gene; MGDG, monogalactosyldiacylglycerol; PhyB, phytochrome B; POR, NADPH–protochlorophyllide oxidoreductase; TLC, thin layer chromatography; 16 : 0, palmitic acid; 18 : 2, linoleic acid; 18 : 3, linolenic acid.

Introduction

Development of the thylakoid membrane is essential to the establishment of photosynthetically fully competent chloroplasts. The thylakoid membrane is the site of photosynthetic electron transport and contains the photosynthetic apparatus, photosystems I and II. Development of thylakoid membranes requires the biosynthesis of lipids. The greater part of thylakoid lipids (almost 80%, w/w) is unique galactolipids, such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (Joyard et al. 1998), which are not seen in large quantities in membranes other than thylakoid membranes. Thus, these galactolipids are thought to determine the physicochemical properties of the thylakoid membrane (Murphy 1982, Murphy 1986), which might be essential in supporting photosynthetic competence.

The final step in MGDG synthesis is catalyzed by MGDG synthase (MGD), UDP-galactose:1,2-diacylglycerol 3-β-D-galactosyltransferase (EC 2.4.1.46) (Joyard et al. 1998). MGD cDNA was cloned first from cucumber (MGD; Shimojima et al. 1997) and subsequently from spinach (soMGD; Miège et al. 1999) and *Arabidopsis thaliana* (atMGD; Awai et al. 2001). DGDG synthesis also requires MGDG as a substrate (Heemskerk et al. 1990, Kelly and Dörmann 2001). Accordingly, MGDG synthesis is considered to be a limiting step for galactolipid biosynthesis, hence the development of chloroplasts.

Recent studies have demonstrated that the biosynthesis of the galactolipids MGDG and DGDG is essential for chloroplast biogenesis, thylakoid membrane formation, and photosynthetic activity. MGDG is found to be a constituent of the crystallographic structure of the reaction centre of PSI and, hence is assumed to play an important role functionally in photosynthesis (Jordan et al. 2001). Genetic evidence also indicates the importance of galactolipid synthesis in the structure and function of chloroplasts. An *Arabidopsis MGD1* mutant (Jarvis et al. 2000) and a DGDG synthase gene mutant (*dgd1*) (Dörmann et al. 1995), which displayed a considerable decrease in MGDG and DGDG levels, respectively, had an abnormal chloroplast ultrastructure as well as reduced photosynthetic activity. In addition to the quantity of the galactolipid, the degree of unsaturation of the galactolipid is known to affect photosynthetic capacity, the chlorophyll content, and photosynthetic

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complexes (McConn and Browse 1998).

Light is known to initiate the greening of etiolated seedlings and galactolipids are reported to accumulate in parallel with greening (Appelqvist et al. 1968a, Appelqvist et al. 1968b, Tevini 1971, Tremolieres and Lepage 1971, Ohta et al. 1995b), although details of the regulation of galactolipid biosynthesis by light are not understood. The authors previously reported that light and cytokinins elevated MGD activity in greening cucumber seedlings (Ohta et al. 1995b). Cytokinin is phytohormone with known promotion of greening. In chlorophyll synthesis, light is essential for the final step catalyzed by NADPH–protochlorophyllide oxidoreductase (POR) (Koski et al. 1951, Griffiths 1978, Apel et al. 1980). In galactolipid synthesis, light may not be essential because galactolipids can accumulate to some extent even in the dark by exogenous cytokinins (Ohta et al. 1995b). Therefore, this cytokinin-dependent increase in MGDG will allow analysis of the independent effects of cytokinins in MGDG synthesis without light. MGDG synthesis may be an indicator for the development of plastids in the dark. Indeed, Chory et al. (1994) reported that cytokinins can induce the development of the thylakoid membrane in the dark probably with galactolipid accumulation.

To clarify the mechanism for regulation of the synthesis of galactolipids during the development of chloroplasts, MGDG synthesis in germinating cucumber seedlings was investigated following illumination and cytokinin treatment with respect to the expression of the *MGD* gene, MGD enzyme activity, the accumulation of MGDG and DGDG, and their fatty acid composition. These experiments used cucumber cotyledons for both biochemical and molecular biological analyzes since larger cotyledons are easy to treat with phytohormone and since the physiological changes of greening can be determined. To examine the effect of cytokinins, cucumber cotyledons were detached from seedlings to interrupt the transport of cytokinins from roots and hypocotyl (Letham 1994). Intact seedlings and detached cotyledons were compared to see the effect of detachment in MGDG synthesis. This study found that light and cytokinins cooperate to regulate the synthesis of MGDG. This paper thus reports the molecular basis of the regulation of galactolipid biosynthesis by light and a cytokinin.

Results

Effects of illumination on MGDG synthesis in cotyledons of intact seedlings

To investigate how light regulates galactolipid synthesis, MGDG synthesis was analyzed with respect to changes in MGD mRNA, changes in MGD activity, the product MGDG, and DGDG synthesized from MGDG during chloroplast development.

The changes in the level of MGD transcripts in intact cucumber cotyledons were first determined. As shown in Fig. 1a, the level of MGD transcripts increased transiently under illumination, whereas the transcripts remained at their initial level in the dark. After 6 h of illumination, the level of MGD transcripts reached the maximum, about fourfold greater than that in the dark. The MGD transcripts then began to decrease, and after 50 h of illumination, the transcript level had returned to the level in cotyledons grown in the dark.

The MGD activity was measured in extracts prepared from cucumber cotyledons illuminated for various periods. As shown in Fig. 1b, a linear increase in enzyme activity with a lag period of 6 h was observed after the onset of illumination. After 50 h of illumination, the activity was twofold greater than that in cotyledons grown in the dark.

The changes in MGDG and DGDG content were measured in parallel. Similar to the profile of changes in the MGD activity, both MGDG and DGDG accumulated simultaneously and gradually in illuminated cotyledons, whereas no increase was seen in cotyledons grown in the dark (Fig. 1c). After 50 h of illumination, MGDG accumulated to more than 75 μg cotyledon^{-1}, which was sevenfold higher than the level in the dark. DGDG also increased more than fivefold. Although the ratio of MGDG to DGDG was around 1.18 to 1.36 in dark-grown cotyledons, the ratio increased to 1.54 at 25 h after the onset of illumination.

Effects of detachment on MGDG synthesis in illuminated cotyledons

As shown in Fig. 1, light induced accumulation of MGD mRNA, resulting in an increase of enzyme activity and MGDG. Another factor known to induce MGDG synthesis are cytokinins (Ohta et al. 1995b), which are thought to be derived from hypocotyl or roots (Letham 1994). To eliminate the effects of any factors transferred from the hypocotyl or roots such as cytokinins, cotyledons were detached at the hook and the effect of illumination in the detached cotyledons was determined.

In detached cotyledons, as shown in Fig. 2a, the level of MGD transcripts rose transiently after 3–9 h of illumination, which was similar to that in intact cotyledons (Fig. 1a) although to a much smaller extent. The enzyme activity increased slightly and was almost equal to the level in the dark. The reduced accumulation of MGD transcripts probably caused the low level of MGD activity.

Although the levels of MGD transcripts and MGD activity did not increase like those in the illuminated intact cotyledons, both MGDG and DGDG accumulated significantly with illumination. MGDG in detached cotyledons accumulated to about 70% of that in intact cotyledons (Fig. 2c). DGDG accumulated in detached cotyledons to almost the same level as in intact cotyledons, indicating that DGDG synthesis was not substantially affected even in the detached cotyledons. The ratio of MGDG to DGDG at 25 and 50 h was 1.08 and 1.14, respectively. These values are smaller than those in intact cotyledons (1.54), indicating that MGDG synthesis was not fully upregulated by illumination in detached cotyledons. The initial enzyme activity in etiolated cotyledons, which is about tenfold higher than that in seeds (Ohta et al. 1995b), probably sus-

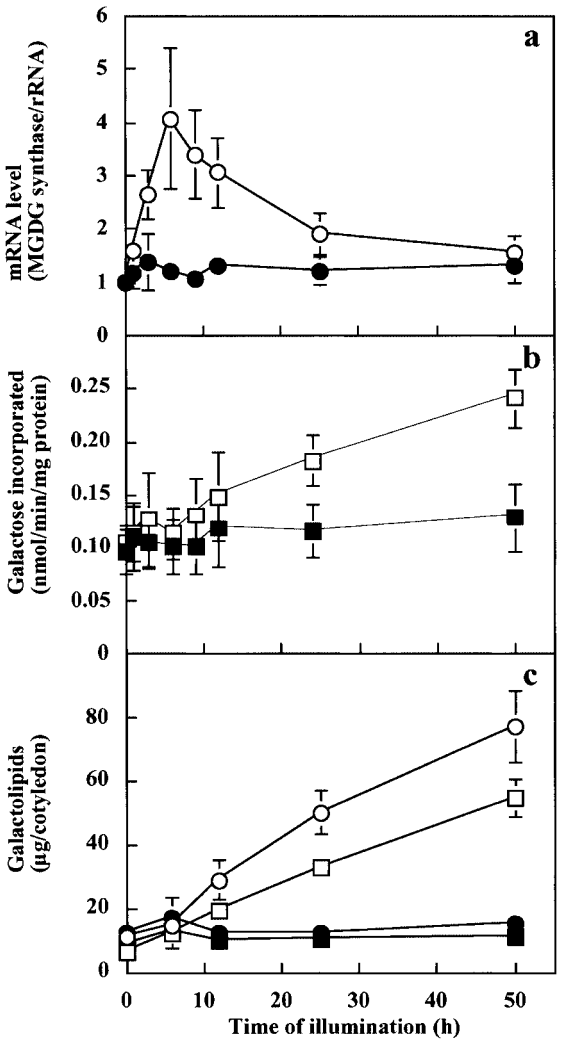

Fig. 1 Effect of illumination on the biosynthesis of galactolipids in intact cotyledons. Germinated cucumber seedlings were illuminated. (a) MGD mRNA level. Illuminated seedlings, (open circles); dark-grown seedlings, (closed circles). (b) MGD activity. Illuminated seedlings, (open squares); dark-grown seedlings, (closed squares). (c) Galactolipid accumulation. MGDG in illuminated seedlings, (open circles); DGDG in illuminated seedlings, (open squares); MGDG in dark-grown seedlings, (closed circles); DGDG in dark-grown seedlings, (closed squares). Each value is the average of three independent experiments. Vertical bars represent standard deviations (SD).

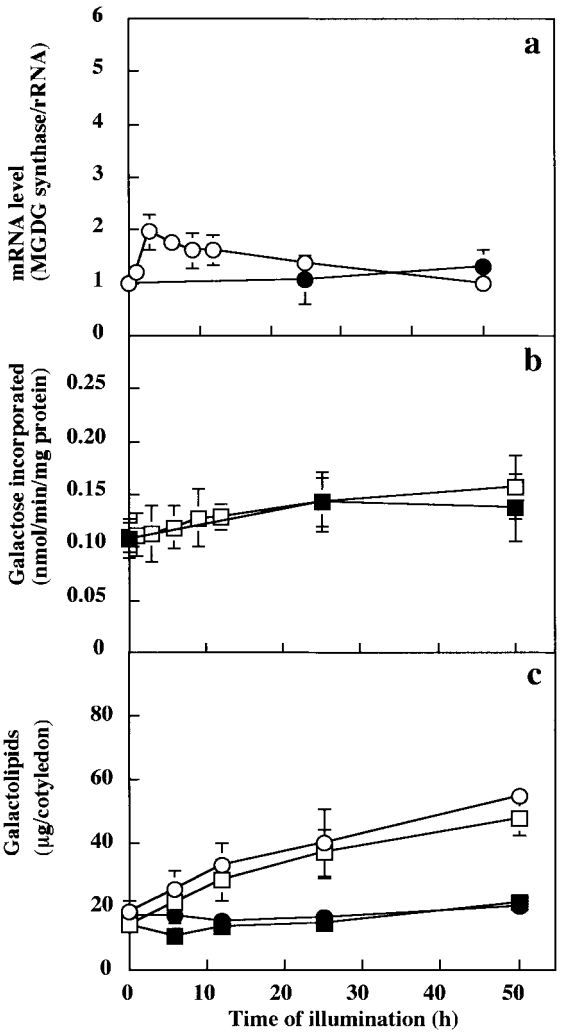

Fig. 2 Effects of excision on the biosynthesis of galactolipids under illumination. Germinated cucumber cotyledons were detached at the hook and illuminated on a dish. (a) MGD mRNA level. Illuminated cotyledons, (open circles); dark-treated cotyledons, (closed circles). (b) MGD activity. Illuminated cotyledons, (open squares); dark-treated cotyledons, (closed squares). (c) Galactolipid accumulation. MGDG in illuminated cotyledons, (open circles); DGDG in illuminated cotyledons, (open squares); MGDG in dark-treated cotyledons, (closed circles); DGDG in dark-treated cotyledons, (closed squares). Each value is the average of results from three independent experiments. Vertical bars represent the SD.

tained the initial accumulation of galactolipids after the onset of illumination or light induced some other factor for the accumulation.

Effects of BA treatment in the dark on MGDG synthesis in detached cotyledons

Since light did not fully activate galactolipid synthesis in detached cotyledons, the detachment possibly stopped transfer of some important factors from the hypocotyl or root to cotyledons. As described above, cytokinins are thought to be derived from hypocotyl or roots (Letham 1994), so the missing factor can be cytokinins. First, the effect of a synthetic cytokinin, benzyladenine (BA) on MGDG synthesis was examined in detached cotyledons in the dark since treatment in the dark enables determination of the sole effects of BA independently of light.

As shown in Fig. 3a, the level of MGD transcripts transiently increased in detached cotyledons after 6 h of BA treatment in the dark, which was similar to the effect of illumination on intact cotyledons (see Fig. 1a). Enzyme activity markedly increased after 12 h of BA treatment in the dark, which was also similar to that in illuminated cotyledons (Fig.

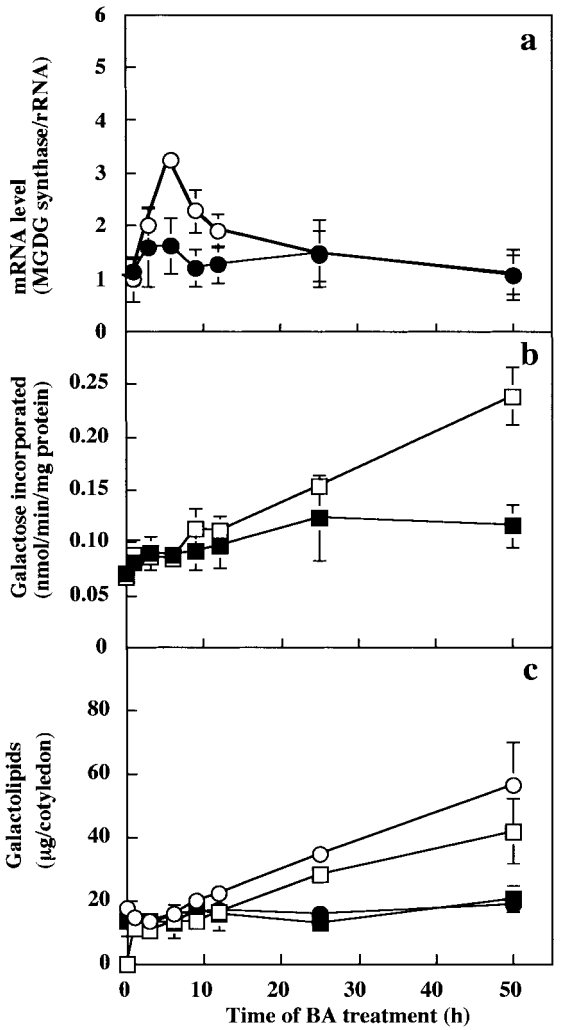

Fig. 3 Effect of BA treatment on the biosynthesis of galactolipids. Germinated cucumber cotyledons were detached at the hook and treated with BA on a dish in the darkness. (a) MGD mRNA level. BA-treated cotyledons, (open circles); water-treated cotyledons, (closed circles). (b) MGD activity. BA-treated cotyledons, (open squares); water-treated cotyledons, (closed squares). (c) Galactolipid accumulation. MGDG in BA-treated cotyledons, (open circles); DGDG in BA-treated cotyledons, (open squares); MGDG in water-treated cotyledons, (closed circles); DGDG in water-treated cotyledons, (closed squares). Each value is the average result of three independent experiments. Vertical bars represent the SD.

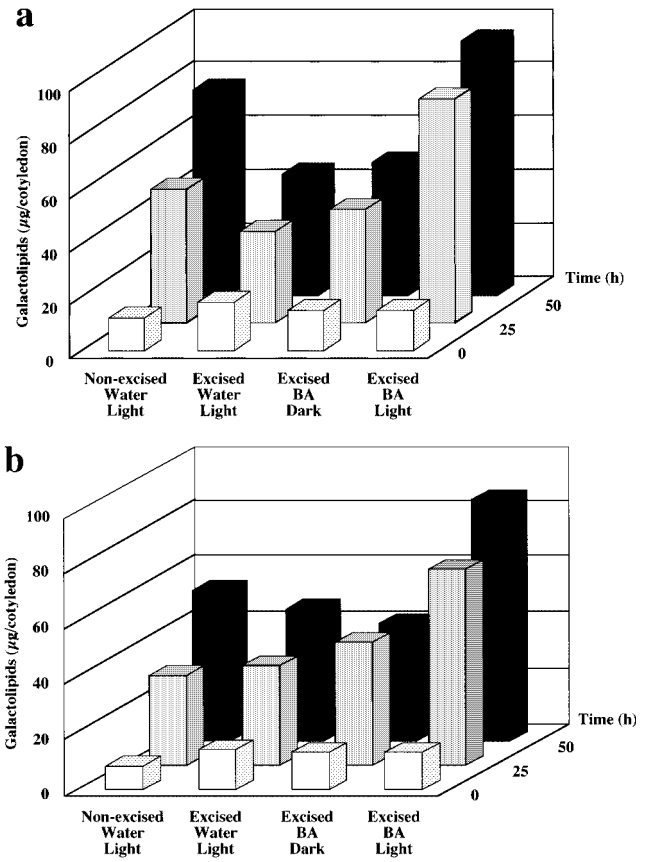

Fig. 4 Effects of co-treatment of BA and light on the accumulation of galactolipids in detached cotyledons. Germinated cucumber cotyledons were detached at the hook and treated with BA and illuminated. (a) MGDG. (b) DGDG. 0 h, open squares, 25 h, shaded squares, 50 h, closed squares. Each value is the average of three independent experiments.

3b). After 50 h of BA treatment, the MGD activity reached twofold the level in water-treated controls. At this point, the enzyme activity was almost equal to that in 50-h illuminated intact cotyledons, even though BA was applied in the dark.

In cotyledons treated with BA in the dark, both MGDG and DGDG increased to about threefold at 50 h when compared with water-treated cotyledons (control) (Fig. 3c). Even in the dark, galactolipids accumulated to some extent after BA treatment. The ratio of MGDG to DGDG at 25 and 50 h after BA treatment was 1.21 and 1.35, respectively. These values suggest that BA enhanced MGDG synthesis by increasing MGD activity. Although BA induced MGD transcript and enzyme activity to a similar extent in intact cotyledons, BA was not a sole factor to maximize the accumulation of galactolipids in detached cotyledons.

Effects of co-treatment of BA and light in detached cotyledons

Since BA induced increases in the level of MGD transcripts and enzyme activity even in detached cotyledons in the dark, whether co-treatment of BA and light could fully restore the accumulation of galactolipids in detached cotyledons to levels that are comparable to those in illuminated intact cotyledons was then examined.

When detached cotyledons were treated with BA and light, MGDG content in detached cotyledons increased more than that in either BA-treated or illuminated detached cotyledons and that in illuminated intact cotyledons (Fig. 4a). DGDG content also increased to a level that exceeded that in illuminated intact cotyledons (Fig. 4b). These results clearly demonstrate that co-treatment of a cytokinin and light fully restored

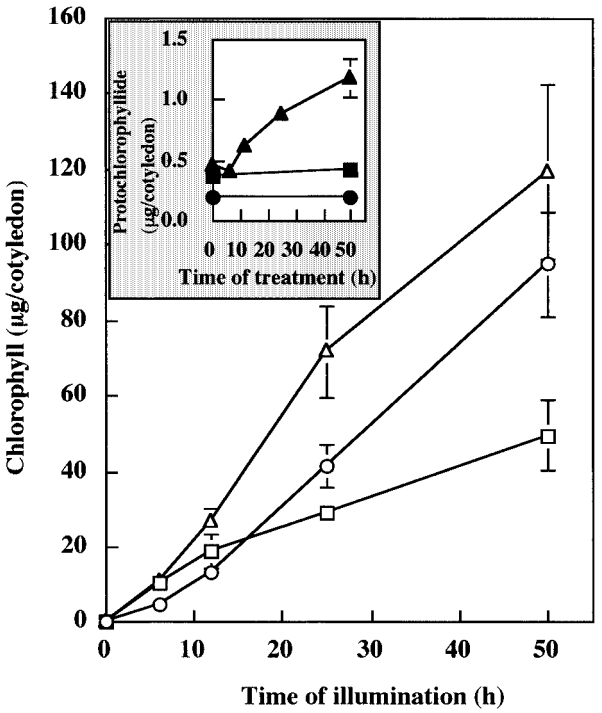

Fig. 5 Change in the chlorophyll content. Intact illuminated cotyledons, (open circles); detached illuminated cotyledons, (open squares); BA and light co-treated detached cotyledons, (open triangles). Protochlorophyllide content in intact cotyledons in the dark, (closed circles); detached cotyledons in the dark, (closed squares); BA-treated detached cotyledons in the dark, (closed triangles). Each value is the average result of more than three independent experiments. Vertical bars represent the SD.

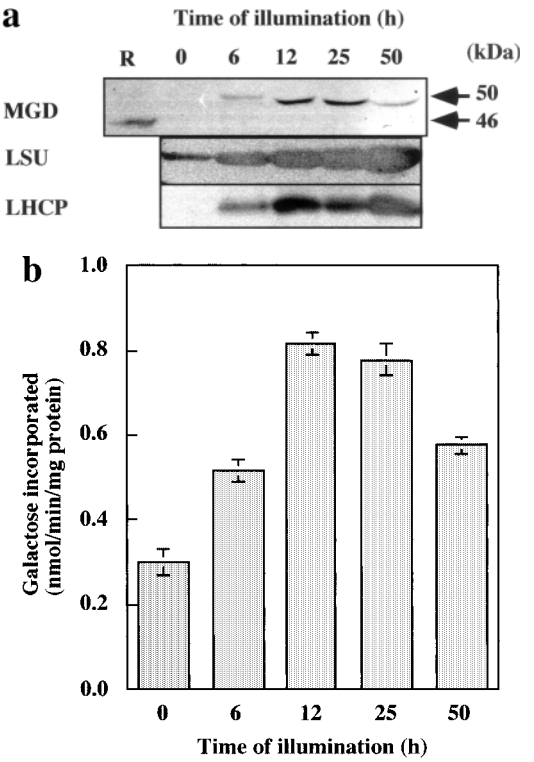

Fig. 6 Effect of illumination on the MGD protein level and activity in the developing chloroplast. (a) Immunodetection of MGD protein in 50 μg chloroplast protein. Recombinant MGD, R; large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, LSU; light-harvesting chlorophyll-binding protein, LHCP. Molecular weight for MGD protein is presented aside. (b) MGD activity in the chloroplast fraction. Each value is the average result of three independent experiments. Vertical bars represent the SD.

the galactolipid accumulation in detached cotyledons to a level comparable to that in intact cotyledons. In addition, MGDG accumulated in cotyledons co-treated with BA and light at a much faster rate than did in illuminated intact cotyledons, suggesting that cytokinin supply (cytokinin biosynthesis or transport to cotyledons) may be one of the rate-limiting steps in galactolipid synthesis during the development stage.

Since BA restored the diminished effect of light with MGDG synthesis in detached cotyledons, the correlation of this effect with BA and chloroplast development was confirmed by the measurement of chlorophyll content (Fig. 5). Light enhanced chlorophyll content concomitantly with illumination time in intact seedlings. In detached cotyledons, chlorophyll content did not increase as much as that in intact cotyledons. In the dark, BA enhanced the accumulation of protochlorophyllide, a precursor of chlorophyll, concomitantly with the treatment time. The chlorophyll content in detached cotyledons increased rapidly with co-treatment of BA and light and more than so that seen in intact illuminated cotyledons, although BA treatment in the dark did not induce chlorophyll at all. These results demonstrated that cytokinin supply affected the level of galactolipids in parallel with the change in chlorophyll content, i.e. the development of chloroplasts.

Effects of light on MGD protein level in chloroplasts isolated from intact cotyledons

As shown in Fig. 1 and 2, light induced MGD transcripts and enzyme activity. The accumulation of the transcript was transient, although the activity increased gradually in cotyledons. Detection of the MGD protein in crude extracts was attempted using an antibody against MGD, but unfortunately the levels of this protein were too low to detect (Maréchal et al. 1994, Ohta et al. 1995a). Only by isolating chloroplasts from intact cucumber cotyledons was the change in MGD protein level detected in developing cucumber seedlings.

As shown in Fig. 6a, MGD protein from chloroplasts was detected as a 50 kDa protein whose molecular mass was higher than that of a deduced mature protein of cucumber MGD (46.6 kDa) (Shimojima et al. 1997). The MGD protein level increased markedly after 6 h of illumination. At 12–25 h, the protein level reached the maximum and dropped at 50 h. In the dark, MGD protein was not detected. The MGD activity was also determined in the chloroplast fraction (Fig. 6b). In the chloroplasts, MGD activity increased after the onset of illumi-

Table 1 Fatty acid composition of MGDG

	Fatty acid, mol% (μg cotyledon^{-1})					
	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	18 : 3
Intact						
Dark 0 h (11.5)	3.66±0.66 (0.421)	1.91±0.56 (0.220)	3.20±0.91 (0.369)	4.49±1.51 (0.516)	16.3±3.61 (1.88)	70.4±2.21 (8.09)
Dark 50 h (15.2)	1.93±0.04 (0.293)	0.67±0.11 (0.101)	1.52±0.26 (0.231)	2.58±0.25 (0.391)	21.2±0.63 (3.22)	72.1±0.03 (10.9)
Light 50 h (76.8)	2.02±0.12 (1.55)	0.50±0.14 (0.382)	0.75±0.12 (0.576)	2.02±0.08 (1.55)	10.6±0.39 (8.16)	84.1±0.33 (64.6)
Detached						
Dark 0 h (17.5)	2.80±0.17 (0.488)	2.35±1.64 (0.410)	1.62±0.53 (0.283)	2.31±0.59 (0.404)	9.70±1.40 (1.69)	81.2±1.07 (14.2)
Dark 50 h (19.7)	2.16±0.31 (0.426)	1.05±0.23 (0.206)	1.88±0.05 (0.370)	2.51±0.18 (0.495)	9.31±1.78 (1.83)	83.1±2.03 (16.4)
Light 50 h (54.2)	1.96±0.25 (1.06)	0.76±0.29 (0.411)	1.36±0.21 (0.736)	2.00±0.28 (1.08)	12.6±0.24 (6.84)	80.2±0.97 (44.0)
BA 0 h (16.2)	2.38±0.41 (0.384)	0.81±0.56 (0.131)	2.51±1.05 (0.406)	3.42±1.03 (0.552)	10.4±1.61 (1.68)	80.4±1.38 (13.0)
BA 50 h (56.2)	1.83±0.32 (1.03)	0.86±0.41 (0.481)	0.84±0.13 (0.472)	1.82±0.23 (1.03)	4.91±0.91 (2.76)	89.7±1.53 (50.4)

Total lipids were extracted from 30 cotyledons collected at the times indicated, and MGDG was separated by two-dimensional TLC. Fatty acid composition was analyzed by gas chromatography. Absolute amount is presented in parentheses.

nation and reached the maximum at 12–25 h in the same manner as the change in the MGD protein level. Due to chloroplast development, major chloroplast proteins, a large subunit of ribulose-1,5-bisphosphatase carboxylase/oxygenase (LSU) and light-harvesting chlorophyll-binding proteins (LHCPs), increased concomitantly with illumination time.

Changes in the fatty acid composition of galactolipids with illumination or BA treatment

The amount of galactolipids and the fatty acid composition of these galactolipids is important for the functioning of chloroplasts. Polyunsaturated fatty acids, the major constituent of galactolipids in developed chloroplasts (Selstam 1998), are essential to maintaining photosynthetic activity (McConn and Browse 1998). To determine the effects of light and a cytokinin on the degree of desaturation in galactolipids, the changes in fatty acid composition of MGDG and DGDG were then analyzed.

In intact cotyledons, light increased the proportion of linolenic acid (18 : 3) in MGDG after 50 h illumination and decreased the proportion of other fatty acids, and especially linoleic acid (18 : 2) (Table 1). In detached cotyledons, 18 : 3 increased proportionally with BA treatment in the dark but not with light. However, 18 : 3 increased in all cases in terms of the absolute amount. The fatty acid composition of DGDG changed similarly. Light enhanced both 18 : 3 and palmitic acid (16 : 0) proportionally in intact cotyledons (Table 2). In detached cotyledons, both BA and light enhanced the proportion of 18 : 3 but not that of 16 : 0. As in MGDG, BA enhanced 18 : 3 production in DGDG.

In both galactolipids, BA probably enhanced desaturation activity directed with respect to 18 : 2. Although detachment caused a higher 18 : 3 content at the basal level, its proportional and absolute increase was marked after BA treatment. Even in the dark, the desaturation of 18 : 2 proceeded without increasing other fatty acids, resulting in a marked proportion of 18 : 3 higher than that in illuminated detached cotyledons. These results imply that both BA and light enhance the enzyme activity of an ω3-desaturase that catalyzes the desaturation of 18 : 2 to 18 : 3.

Expression of MGDG synthase in the lh mutant

Since light is required for the accumulation of MGDG, the photoreceptor responsible for the accumulation of MGD mRNA was analyzed. Light-dependent expression of photosynthesis-related genes is generally mediated by the photoreceptor phytochrome (Kuno et al. 2000). Therefore, the photoreceptor for MGD was expected to be phytochrome and the light-dependent accumulation of MGD mRNA in the long hypocotyl (*lh*) mutant, which has a mutation in phytochrome B (PhyB) (López-Juez et al. 1992), was analyzed.

In intact cotyledons from the *lh* mutant, MGD mRNA was detected at levels identical to that in the wild type (Fig. 7a, b, white light). These results indicate that PhyB is not required for the light-dependent induction of the MGD gene.

Effects of far-red light >700 nm on galactolipid synthesis

With cucumbers, the *lh* mutant was only the mutant avail-

Table 2 Fatty acid composition of DGDG

	Fatty acid, mol% (μg cotyledon^{-1})					
	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	18 : 3
Intact						
Dark 0 h (7.30)	8.68±0.87 (0.633)	3.34±1.85 (0.244)	7.74±0.40 (0.565)	6.45±1.53 (0.470)	11.7±1.99 (0.855)	62.0±4.54 (4.53)
Dark 50 h (11.2)	8.19±0.12 (0.917)	1.11±0.26 (0.125)	7.06±0.85 (0.791)	3.32±0.28 (0.372)	10.9±0.37 (1.22)	69.4±0.05 (7.78)
Light 50 h (54.3)	11.7±0.13 (6.41)	0.75±0.16 (0.408)	4.11±0.10 (2.23)	2.34±0.07 (1.27)	5.19±0.09 (2.82)	75.8±0.09 (41.2)
Detached						
Dark 0 h (14.0)	9.40±0.35 (1.32)	1.89±0.48 (0.265)	7.49±1.04 (1.05)	3.51±0.36 (0.490)	9.15±0.97 (1.28)	68.5±1.61 (9.59)
Dark 50 h (20.9)	8.28±0.27 (1.73)	2.64±1.02 (0.553)	7.40±0.99 (1.55)	3.82±0.54 (0.798)	6.81±1.50 (1.42)	71.1±1.90 (14.8)
Light 50 h (47.6)	10.4±0.27 (4.97)	1.05±0.33 (0.498)	5.75±0.65 (2.74)	2.93±0.82 (1.39)	6.42±0.90 (3.05)	73.4±1.68 (34.9)
BA 0 h (13.2)	8.79±0.79 (1.16)	1.32±0.98 (0.175)	7.42±0.38 (0.979)	4.80±0.74 (0.634)	10.4±1.18 (1.38)	67.2±2.37 (8.88)
BA 50 h (41.7)	8.40±0.54 (3.50)	0.87±0.23 (0.361)	4.78±0.45 (1.99)	2.55±0.40 (1.07)	2.39±0.39 (0.996)	81.0±1.70 (33.8)

Total lipids were extracted from 30 cotyledons collected at the times indicated, and DGDG was separated by two-dimensional TLC. Fatty acid composition was analyzed by gas chromatography. Absolute amount is presented in parentheses.

able to determine the photoreceptor. In order to verify a possible photoreceptor, PhyA, light sources with wavelengths restricted to either over 600 nm (red light) were used for both PhyA and PhyB or over 700 nm (far-red light) was used for PhyA. Far-red light readily induced the accumulation of MGD mRNA in the wild type and even in the mutant (Fig. 7a, b), suggesting that the possible photoreceptor is PhyA.

Far-red light enhanced enzyme activity to almost the same level as that induced by white light (Fig. 8), but galactolipids accumulated to less than half the level with white light, while red light induced galactolipid accumulation similar to white light (Fig. 9a, b). This strongly suggests that red light (600–700 nm) is required for the normal accumulation of galactolipids.

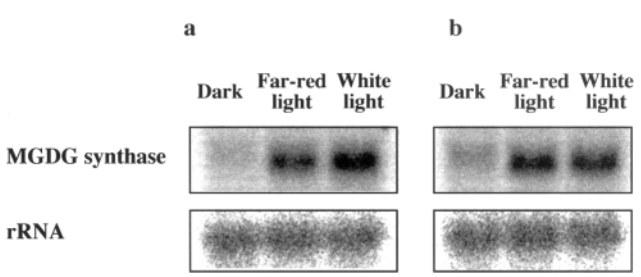

Fig. 7 Effects of red or far-red light on the MGDG synthase mRNA level in *lh* mutant. Far-red light (>700 nm) or white light irradiation for 7.5 h on (a) wild-type seedlings, (b) *lh* mutant seedlings (López-Juez et al. 1992).

Discussion

Regulation of MGDG synthase expression

This series of experiments examined the effects of light and BA on galactolipid synthesis using intact and detached cucumber cotyledons. Analysis of the levels of MGD transcripts and enzyme activity and of lipid composition indicated

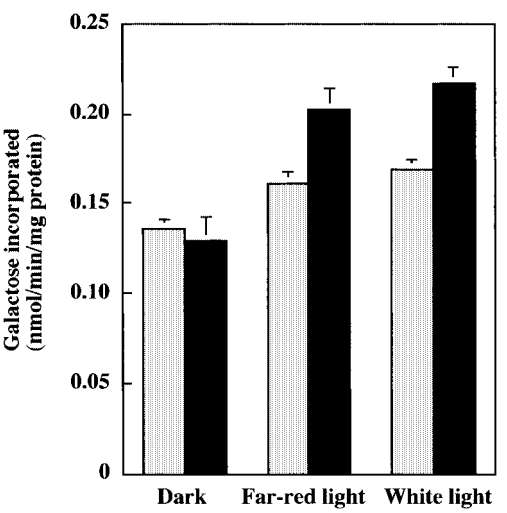

Fig. 8 Effect of far-red light on the MGDG synthase activity. Far-red light (>700 nm) or white light irradiation on seedlings. 25 h, shaded squares, 50 h, closed squares. Each value is the average of three independent experiments. Vertical bars represent the SD.

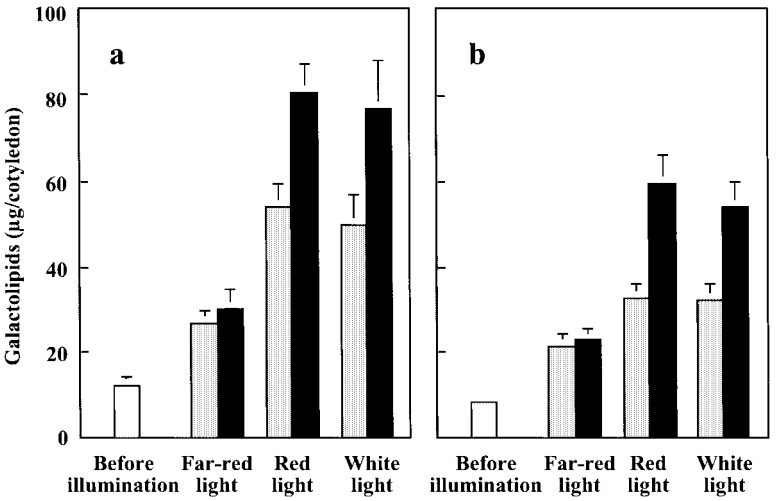

Fig. 9 Effects of far-red light or red light on the accumulation of galactolipids. Far red light (>700 nm), red light (>600 nm) or white light on irradiation seedlings. (a) MGDG; (b) DGDG; 0 h, open squares, 25 h, shaded squares, 50 h, closed squares. Each value is the average of three independent experiments and vertical bars represent the SD.

that light and a cytokinin are regulatory factors for MGDG synthesis. Light also induced early accumulation of MGD protein in isolated chloroplasts. In isolated chloroplasts, MGD protein and activity increased transiently (Fig. 6a, b) although MGD activity in the total protein fraction gradually increased (Fig. 1b). This difference indicates that MGD protein increased rapidly only in the early stage of chloroplast development. In the later stage, accumulation of major chloroplast proteins may overcome the increase in MGD protein. During the development of chloroplasts, MGD protein accumulated as fast as LHCPs and earlier than a major stromal protein, LSU. This suggests that the early synthesis of MGDG and DGDG is important for proper accumulation of thylakoidal proteins such as LHCPs. Since the change in the accumulation of galactolipids (Fig. 1c, 2c, 4a, 4b) agreed with the change in the level of chlorophyll (Fig. 5), the galactolipid accumulation coordinated well with the development of chloroplast. The results imply that the galactolipid accumulation induced by BA in the dark also coordinates with the development of plastids although chlorophyll did not accumulate. In fact, exogeneous cytokinin induced the development of thylakoid membrane in the dark (Chory et al. 1994). Therefore, the level of galactolipids accumulation will be a good indicator for the chloroplast development both in the light and in the dark.

During galactolipid synthesis in greening cotyledons, light first triggered a transient increase in MGD transcripts, which was followed by the accumulation of MGD protein and the enhancement of enzyme activity. These sequential events resulted in the accumulation of both MGDG and DGDG. Meanwhile, desaturation activity also increased and resulted in a high proportion of 18 : 3 in the MGDG and DGDG of cotyledons, as in mature leaves (Selstam 1998). Since MGD gene expression did not depend on PhyB, some other photoreceptor, probably PhyA, may be responsible for the expression. Research must proceed further to determine the responsible photoreceptor using available *Arabidopsis* mutants in photosignal transduction.

Cytokinin plays a co-operative role with light in MGDG synthesis

Light affected MGDG synthesis differently in intact and detached cotyledons, and especially in transcript levels and enzyme activity; therefore, light was not the sole factor for MGDG synthesis as a whole to proceed. Since detachment did not affect the levels of MGD transcripts or enzyme activity in the dark, the deficient factor(s) induced by light and delivered from the hypocotyl or roots would be responsible for the differences and the limited accumulation of MGDG. In detached cotyledons, BA raised the level of MGD transcripts and enzyme activity even in the dark but galactolipids accumulated only to slight levels (Fig. 3). Light induced galactolipid accumulation in detached cotyledons to 70% of that in intact cotyledons but did not induce an increase in MGD mRNA and enzyme activity (Fig. 2). MGDG accumulated fully in detached cotyledons with co-treatment of BA and light, and MGDG synthesis was restored to the level in intact greening cotyledons (Fig. 4a). Thus major deficient factor derived from hypocotyl or roots should be a cytokinin. In cucumber cotyledons, cytokinins regulate MGDG synthesis in co-operation with light for the development of chloroplasts.

To examine the direct involvement of cytokinins in light signal transduction, intact cotyledons were treated with a cytokinin antagonist, 2-chloro-4-cyclobutylamino-6-ethyl-amino-s-triazine (CCET). Although CCET is a recognized cytokinin antagonist (Sano and Youssefian 1994), the expansion of the cotyledons by BA was not inhibited in detached cucumber cotyledons (data not shown). In intact cotyledons, changes due to CCET were not noted in either the induction of MGD transcripts or galactolipid accumulation. A direct link between light and a cytokinin remains to be demonstrated with a more effective cytokinin antagonist.

Cytokinins induce desaturation of fatty acids similarly to light even in the dark

Light and BA similarly enhanced the desaturase activity of fatty acids in MGDG and DGDG. The proportion of 18 : 3 of both MGDG and DGDG rose after the onset of illumination or BA-treatment in the dark. An increase in polyunsaturated fatty acids, and especially 18 : 3, agrees with the theory that light and BA induce the development of the thylakoid membrane, since polyunsaturated fatty acids are essential for chloroplast development and eventual photosynthetic functioning (McConn and Browse 1998). Although many groups have reported an 18 : 3 increase in galactolipids during the greening step (Selstam 1998), the current work is the first time that a cytokinin has induced an 18 : 3 increase similar to that of light.

The estimation of mature protein size for MGD

In Western blotting analysis, the MGD protein from chloroplast had a different molecular weight from that of rMGD protein (Fig. 5a). The N terminus of the mature MGD protein, Gly-104, was identified from purified cucumber MGD protein (Shimojima et al. 1997). An rMGD protein was therefore constructed from Gly-104 to Gly-525 (421 aa) with a molecular mass of about 46.6 kDa. MGD protein from chloroplasts, however, had a molecular mass of about 50 kDa. MGD protein can be modified post-translationally, but the mature region of the MGD protein can more likely be longer than what Shimojima et al. (1997) deduced before.

The latest prediction server for chloroplast transit peptides and their cleavage sites, ChloroP (Emanuelsson et al. 1999) predicted that Leu-68 for the cleavage site of MGD protein resulting in the MGD mature region would be 457 aa, 50.4 kDa. This molecular mass is similar to that of *Arabidopsis MGD2* and *MGD3*, which probably do not have transit peptides. The purified MGD protein reported by Shimojima et al. (1997) may have lost 36 residues at the N terminus during the purification. Mature MGD protein will be longer than 46 kDa, and probably around 50 kDa, based on current results of Western blotting and processing site predictions.

Another role of light for MGDG biosynthesis

Both red (>600 nm) and far-red light (>700 nm) induced the increase in MGD gene expression and enzyme activity. However, far-red light resulted in a slight accumulation of galactolipids, while red light (>600 nm) caused the full accumulation of galactolipids to a level as high as with white light. Obviously, red light (600–700 nm) is essential for MGDG synthesis apart from the induction of gene expression or an increase in enzyme activity. In fact, light can enhance galactolipid accumulation in detached cotyledons without increasing the level in MGD transcripts and activity (Fig. 2). Therefore, several other factors induced by red light but not by far-red light are required for sufficient galactolipid accumulation.

The first possibility is that the galactolipid synthesis requires the synthesis of other proteins and pigments, which are probably essential for the proper assembly of the photosynthetic membranes in vivo. Inhibition of de novo protein synthesis by cycloheximide completely blocked increases in MGD activity and galactolipids (Ohta et al. 1995b). Therefore the light-dependent accumulation of galactolipids requires some protein synthesis other than MGD.

The second possibility is that the synthesis of substrate fatty acid requires red light (600–700 nm) because the key enzyme of fatty acid synthesis, acetyl-CoA carboxylase, is regulated by light via a redox cascade (Sasaki et al. 1997). To investigate whether substrate supply is required to induce sufficient accumulation of galactolipids, the effect of cerulenin, an inhibitor of fatty acid synthesis (D'Agnolo et al. 1973, Omura 1976), was examined. In cerulenin-treated cotyledons, both MGDG and DGDG accumulated to the same extent as water-treated cotyledons (data not shown). Therefore, de novo diacylglycerol (DG) synthesis is not necessary as a substrate for cotyledon development.

Laskay et al. (1984) reported that detached barley leaves that were treated with cerulenin, however, contained reduced amounts of the lipids MGDG and DGDG. Because barley leaves do not contain substantial storage lipids, unlike oil-seed plants, de novo fatty acid synthesis is necessary. In cucumber cotyledons, the degradation of storage lipids is an important process in germination, so fatty acids or DG are probably rich enough for galactolipid synthesis, and especially in this early developmental stage (Feussner et al. 2001).

A final possibility is that MGDG synthesis in vivo is regulated in co-operation with the expression of photosynthetic activity. Red light caused cucumber cotyledons to green normally, and the expression of both MGD and MGD enzyme activity was similar to that with white light (data not shown). Although far-red light increased MGD mRNA levels similar to that with white light (Fig. 7a), greening did not occur. This phenomenon reflects the strict requirement for red light around 660 nm for the light-dependent reactions of POR in chlorophyll synthesis (Koski et al. 1951, Griffiths 1978, Apel et al. 1980). These facts indicate the possibility that sufficient accumulation of galactolipids requires expression of photosynthetic activity that is not mediated merely by the irradiation of far-red light.

MGD activity itself was recently found to be regulated by redox potential in vitro (Yamaryo et al. 2003) like the activity of acetyl-CoA carboxylase (Sasaki et al. 1997). The limited accumulation of galactolipids in far-red light or BA in the dark might be partially due to a lack of light-dependent electron transport via chlorophyll. Full MGD activity can be detected only in reducing conditions in vitro. In fact, MGD activity strictly requires reducing compounds such as DTT (Covès et al. 1986), suggesting that MGD activity is regulated by redox potential in vivo. The authors are currently examining in detail the effects of redox potential on the activation of MGD.

Materials and Methods

Preparation of plant material

Cucumber seeds (*Cucumis sativus* L. cv. Aonagajibai) were sown on wet vermiculite, and grown at 27°C in the dark. After 4 d, the cotyledons were cut off at the end of hook and treated with water on a dish containing a piece of paper (3MM Cr, Whatman, U.K.) for 12–15 h to eliminate unexpected changes induced by excision ("detached cotyledons"). Cotyledons were then treated with cytokinin, BA, or illumination (white fluorescent light: 23–50 μmol m^{-2} s^{-1}). Ten cotyledons were used to evaluate mRNA levels, 20 for the enzyme assay, and 30 for lipid analysis, and were collected at the times indicated. For BA treatment, BA solution was added to the dish to a final concentration of 100 μM, after the elimination of unexpected changes induced by excision.

We prepared another set of etiolated seedlings, which were illuminated directly, 4 d after germination in the dark, as described above, and the cotyledons were detached just before analysis ("intact cotyledons"). Filters that restrict the light to more than 600 nm and more than 700 nm were used for red- and far-red-light irradiation, respectively (red light by halogen lamp: 48–60 μmol m^{-2} s^{-1}; far-red light by halogen lamp: 0.05–0.10 μmol m^{-2} s^{-1}). For treatment with cerulenin (Sigma, U.S.A.) or cytokinin antagonist (a gift from Prof. Iwamura), cucumber seeds were sown on 18 ml of 0.3% agarose in a plant box (Iwaki, Japan) and grown at 27°C in the dark. Cerulenin dissolved in ethanol was applied to a final concentration of 100 μM just before the cotyledons were illuminated. Ethanol (0.1%) was used as the control. The cytokinin antagonist, CCET, was dissolved in DMSO and applied at a final concentration of 50 μM. DMSO (12.5 μl) was used as the control. Samples were incubated for 24 h in the dark and then illuminated.

Northern hybridization

Total RNA (10 μg $lane^{-1}$) was isolated by the method of Chomczynski and Sacchi (1987), denatured, subjected to electrophoresis on 1.2% agarose gel, and transferred to a nylon membrane. The blot was hybridized with a α-[^{32}P]dCTP-labeled full-length MGD cDNA (2,142 bp) at 65°C. After hybridization, the blots were washed with 2× SSC (3 M NaCl in 0.3 M sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate (SDS) for 5 min followed by two washes in 0.1× SSC/0.1% SDS for 20 min. The membrane was exposed to an imaging plate (Fuji Photo Film, Japan), and analyzed with a BAS 2000 analyzer (Fuji Photo Film, Japan).

Assay for MGDG synthase activity

An enzyme mixture for the assay was prepared from an extract of cucumber cotyledons. Twenty cotyledons were macerated with a mortar and a pestle with five volumes of MSPB buffer (50 mM 3-morpholinopropanesulfonic acid [MOPS]–NaOH [pH 7.9], 10 mM DTT, 0.8 M sodium acetate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 20% glycerol [v/v], 0.02% NaN_3 [w/v], and 10 μM leupeptin). Samples were centrifuged at 500×*g* for 20 min, and the supernatant used as the enzyme mixture. MGD activity was measured by the amount of [4,5-^{3}H]galactose incorporated into the lipid fraction, according to the method of Teucher and Heinz (1991). The prepared enzyme mixture (30 μl) was mixed with 50 μl 1,2-dioleoyl-*sn*-glycerol (200 μg dispersed in 0.01% Tween-20), and diluted to 190 μl with MOD buffer (100 mM MOPS–NaOH [pH 7.8], 3 mM DTT). The procedure followed was that of Ohta et al. (1995b), except that the incubation time was 20 min. Proteins were quantified by the method of Bensadoun and Weinstein (1976), with bovine serum albumin as the standard.

Measurement of galactolipid content

Total lipids were extracted by the method of Bligh and Dyer (1959) from 30 cotyledons of cucumber seedlings. MGDG and DGDG were then separated by thin layer chromatography (TLC) on silica gel plates (60 F_{254}, Merck, Germany), and developed with a mixture of chloroform, methanol, and water (65 : 15 : 2, v/v). Each lipid was extracted from the silica gel scraped from the TLC plate and the levels of galactolipids were determined spectroscopically by the anthrone–sulfuric acid method of Radin et al. (1955).

Analysis of fatty acid composition

Total lipids were extracted from cucumber cotyledons as described above, and separated chromatographically in two dimensions on silica gel plates (60 F_{254}, Merck, Germany), using the following solvent systems: chloroform–methanol–7 M ammonia (15 : 10 : 1, v/v) in the first dimension and chloroform–methanol–acetic acid–water (170 : 20 : 17 : 3, v/v) in the second dimension. Total lipids were sprayed with 0.01% primuline in 80% acetone and visualized under UV light. MGDG and DGDG were extracted from the silica gel scraped from the TLC plate. Fatty acid compositions of MGDG and DGDG were determined by gas chromatography (GC14A gas chromatograph, Shimadzu, Japan; HR-SS-10 capillary column, 25 m × 0.25 mm, Shinwa Chemical Industries, Ltd., Japan) after derivatization with 5% (v/v) HCl in methanol.

Assay for chlorophyll content

Chlorophyll or protochlorophyllide content was measured in a pair of cucumber cotyledons for various times (0, 6, 12, 25, 50 h) following the method described by Arnon (1949) and Anderson and Boardman (1964), respectively.

Isolation of chloroplast

Chloroplasts were isolated from ~10 g of cucumber cotyledons of seedlings illuminated for various time (0, 6, 12, 25, 50 h) following the method described by Douce and Joyard (1982).

Construction of MGD expression vector

The deduced mature region of MGD cDNA (from Gly-104 to Gly-525) was amplified using PCR primers 5′-GATATCATGGTTTC-AGATGAAACCAATGGG-3′ and 5′-GATATCTCAGCCGGAATATT-GTGGTAC-3′. The MGD cDNA was subjected to 30 cycles of PCR amplification (0.5 min at 94°C, 0.5 min at 55°C, and 1 min at 72°C) using *Takara Ex Taq* (TaKaRa, Otsu, Japan) according to the manufacturer's instructions. The amplified product was ligated into pPICT-2 (Kashima et al. 1999) and digested with EcoRV. The obtained fragment was ligated into expression vector pET 24a (Novagen) followed by the transformation of this vector to *Escherichia coli* DE3.

Antibody preparation

A rabbit was immunized with ~100 μg of purified MGD from GST-fusion protein (Shimojima et al. 1997) with complete or incomplete adjuvant (Nakarai Tesque, Kyoto, Japan). The obtained antiserum was used for Western blotting analysis.

Western blotting analysis

Proteins of the chloroplast fraction (50 μg) was precipitated with 80% acetone and separated on SDS-PAGE gels and then electroblotted onto nitrocellulose membranes (Schleicher & Schuell). The membranes were incubated with the cucumber MGD antibodies followed by the incubation with the horseradish peroxidase-conjugated anti-rabbit IgG (anti-rabbit IgG (H+L); Vector Laboratories, Burlingame, CA, U.S.A.). The bands of cucumber MGD were detected using ECL plus Western blotting detection reagents (Amersham Bioscience, Inc.).

Acknowledgments

We would like to thank Prof. RE Kendrick for providing us *lh* mutant of cucumber and also thank Prof. H Iwamura for providing us a cytokinin antagonist, CCET. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (No.09274211, 10170211, and 11151208 for Takamiya K, and 10178203 and 11640644 for Ohta H) from the Ministry of Education, Sports, Science and Culture, Japan.

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(Received February 27, 2003; Accepted June 23, 2003)