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GALACTOLIPID BIOSYNTHESIS IN *ZEA MAYS* SHOOTS

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Abstract—Illumination of dark-grown maize seedlings produced an increase in their mono- and di-galactosyl contents with the two galactose moieties being donated by UDP-galactose. Diolein was an effective exogenous acceptor of the first galactosylation and endogenous maize mono-galactosyl diglyceride of the second.

INTRODUCTION

MONO- and di-galactosyl diglycerides, first identified in wheat flour,[1–3] are now generally regarded as important components of the photosynthetic systems of all organisms except the bacteria.[4] Chloroplast membranes contain the largest quantities[5] but they have also been detected in other membrane systems.[6] It has been suggested that they are involved in fatty acid synthesis,[7] sugar transport across chloroplast membranes[8] and a largely structural role with their acyl chains interlocking with the phytol groups of chlorophyll molecules.[7,9] The high proportion of α-linolenic acid in chloroplast lipids is largely due to its presence in galactolipids.[4,10–12]

Isolated chloroplasts and chloroplast extracts have been shown to catalyse the synthesis of galactolipids from UDP-galactolipids.[13–17] Acetone powder preparations of spinach chloroplasts were found to synthesise galactolipids when supplied with certain diglycerides whilst those of grass species did not.[15]

RESULTS AND DISCUSSION

Galactolipids in the Greening Up of Maize Shoots

Maize seeds were germinated in darkness for 7 days. Half of these seedlings were grown on in constant warm white fluorescent light and the other half kept in darkness. Both light and

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dark grown seedlings were sampled at intervals over 3 days and the chlorophyll and glycolipid contents of their shoots determined. Most of the changes associated with greening up took place during the first 12–24 hr, with the exception of chlorophyll development which went on throughout the 72 hr of illumination (Table 1).

TABLE 1. CHANGES IN THE LIPID FRACTIONS DURING THE GREENING OF 7-DAY-OLD DARK GROWN MAIZE SEEDLINGS (mg/g dry wt)

Hours of illumination	Chlorophyll	Total lipid	Neutral and phospholipid fraction	Glycolipid fraction	Total	Glycolipid Monogalactosyl	Galactose Digalactosyl
0	0·05	58·1	34·2	22·4	0·74	0·19	0·55
6	0·63	58·0	32·5	25·1	0·92	0·32	0·60
12	1·42	72·2	39·3	30·9	1·13	0·43	0·70
24	2·32	74·4	42·5	30·0	2·00	0·77	1·23
48	3·72	81·8	45·6	32·1	2·17	0·82	1·35
72	4·49	85·4	52·6	31·5	2·24	0·82	1·42
72 darkness	0·05	78·1	49·2	26·3	0·73	0·19	0·54

The total lipid fraction increased in both light and darkness through the 72 hr of the experiment. However, the glycolipid fraction from the illuminated seedlings increased during the first 12 hr of illumination thereafter remaining steady. The glycolipid fraction from the darkened seedlings increased much less. The neutral lipid–phospholipid fraction showed an initial transient decrease at 6 hr but increased for the remainder of the 72 hr. It seems likely that this was due to a small part of the neutral lipid–phospholipid fraction being utilized in glycolipid synthesis, for example, as α-linolenate.[7,9]

The total glycolipid–galactose content of the illuminated seedlings increased almost three-fold during the first 24 hr of greening and then remained almost constant. There was virtually no increase in the glycolipid–galactose content of darkened seedlings indicating that the slight increase in the glycolipid fraction in the dark, mentioned above, was not due to an increase in galactolipid but to some other components of the fraction.

Thus, virtually all the increase in galactolipid took place during the first 24 hr of illumination and as a consequence of it, while chlorophyll development continued non-synchronously throughout the 72 hr of illumination. There appears to be considerable variation possible as synchronous galactolipid and chlorophyll synthesis has been reported in *Euglena*,[6,18] and no light requirement reported in *Scenedesmus*.[18] Both mono- and di-galactosylyl diglyceride contents increased during the first 24 hr of illumination; the proportion of digalactosyl diglyceride decreasing over this period from about 59 to 46% of the total galactolipid. This is also a different pattern from *Chlorella* and *Euglena*[18] where the monogalactosyl diglyceride was found to be the major component and, in the case of *Chlorella*, the only one to increase on greening.

The largest fatty acid component of the total maize galactolipids (Table 2) was α-linolenic acid which made up about 30% of the galactolipid fatty acid in dark-grown seedlings and 65% in greened seedlings. This large increase took place entirely during the first 24 hr of illumination. The most important of the remaining minor fatty acids were palmitic, linoleic and oleic acids. The pattern was substantially the same in both mono- and di-galactosyl

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diglycerides, except that the mono- were more unsaturated than the di-galactosyl diglycerides due to somewhat higher proportions of linolenic and linoleic acids. Similar differences have been reported for spinach[13–16] and algal galactolipids.[18] The C_{16} polyenoic acids reported could not be detected.

TABLE 2. THE FATTY ACID COMPOSITION % OF TOTAL MAIZE SHOOT GLYCOLIPIDS DURING GREENING

Fatty acid	Duration of illumination (hr) 0	6	12	24	48	72	Control
12:0	0·18	0·13	0·13	0·25	0·34	0·36	0·12
12:–	0·06	0·06	0·07	0·16	0·21	0·21	0·04
14:0	0·27	0·18	0·34	0·37	0·11	0·15	0·31
14:–	0·04	0·06	0·06	0·10	0·15	0·10	0·08
15:0	0·16	0·11	0·25	0·29	0·15	0·12	0·21
16:0	23·73	22·21	19·19	18·72	18·01	15·41	25·44
16:1	0·76	0·49	0·56	0·38	0·54	0·52	0·61
16:2	0·24	0·29	0·25	0·27	0·48	0·38	0·40
18:0	2·29	1·92	2·24	1·31	1·46	1·81	1·98
18:1	3·66	2·57	2·33	1·90	1·80	1·61	3·77
18:2	36·19	34·85	27·47	22·11	21·03	15·36	36·57
18:3	32·42	37·13	47·11	54·14	55·72	63·97	30·47

These differences in occurrence and properties suggest the possibility of differing roles for mono- and di-galactosyl diglycerides.

Incorporation of [^{14}C]-*Galactose into the Lipids of Maize Shoots during Greening Up*

Maize was germinated in darkness for 7 days, the shoots cut off and their cut ends placed in a solution of UDP-[^{14}C]-galactose. Eight batches were then illuminated for periods of 0·5–72 hr and two other batches for comparison were given respectively 48 hr continued darkness, and 24 hr illumination followed by 48 hr of darkness. At the end of these treatments the lipids were extracted and separated and their radioactivity estimated.

TABLE 3. INCORPORATION OF UDP-[^{14}C]-GALACTOSE DURING THE GREENING OF 7-day-old DARK-GROWN MAIZE SHOOTS

Duration of illumination (hr)	Shoots (Hz)	Total lipid (Hz)	Galactosyl diglycerides (Hz) Mono-	Di-
0·5	8574	210	60	90
1	9332	390	90	150
3	9455	1890	510	780
6	9963	4820	1280	2040
12	10 006	10 530	2610	3630
24	10 079	26 220	8910	8370
48	10 233	25 810	7560	8550
72	10 255	25 200	4890	8940
24 + 48 darkness	10 257	25 120	6090	8490
48 darkness	10 051	5730	210	840

Uptake of label into the shoots was rapid (Table 3), reaching 80% of the available radioactivity in 30 min then increasing more slowly to 96% after 48 hr of illumination. Incorporation into the lipid fraction was much slower, only 0·04% being incorporated in the first

30 min but after 24 hr illumination, incorporation had increased 100-fold to 4·34%. The shoots grown without illumination took up label just as readily as illuminated shoots but incorporated very little of this label into lipid. Thus illumination had little influence on uptake of UDP-galactose into the shoot but promoted its incorporation into lipid.

Labelled galactolipids increased rapidly during the first 24 hr of illumination reaching 65% of the radioactivity in the lipid fraction and for the first hour all the galactolipid label was present in the galactolipid galactose. Both labelled mono- and di-galactosyl glycerides increased at the same rate but neither increased to any extent in the dark-grown seedlings. These results suggest that UDP-galactose donated galactose during the synthesis of both mono- and di-galactosyl glycerides.

Longer periods of illumination resulted in little increase in the count rate of digalactosyl glycerides but there was a significant fall in the count rate of monogalactosyl glycerides. This loss may have been due to galactolipid being broken down by enzymes such as the β-galactosidase in spinach leaves,[19] being involved in fatty acid synthesis as suggested by Nichols[7] or conversion into digalactosyl diglyceride.

Tri- and tetra-galactosyl glycerides. such as those in spinach leaves,[13,14] were not detected.

TABLE 4. DISTRIBUTION OF LABEL FROM UDP-[^{14}C]-GALACTOSE BETWEEN GALACTOLIPIDS AFTER INCUBATION WITH MAIZE-CHLOROPLAST-ACETONE POWDER AND SUBSTRATES

Substrate	Incorporation into total lipid % of applied label	Incorporation into total galactolipid % of applied label	Label in glycolipid fractions % of total glycolipid	
			Monogalactosyl	Digalactosyl
None	2·0	0·0	0	0
Chloroplast filtrate	19·5	18·4	86	14
Maize mono-galactosyl diglyceride	27·6	25·8	5	95
Triolein	1·7	0·0	0	0
Trilinolenin	2·1	0·0	0	0
Diolein	24·0	23·0	96	4
Dipalmitin	2·3	0·0	0	0

Incorporation of [^{14}C]-*Galactose into the Lipids of Maize Chloroplasts*

An acetone powder preparation of fully greened up maize chloroplasts was prepared in order to separate the chloroplast enzymes from the naturally occurring galactose acceptors.[13,14] The chloroplasts acetone powder was mixed with a substrate and UDP-[^{14}C]-galactose then incubated in Tris buffer at pH 7·4 for 1 hr at 37° in the light. The following seven substrates were used separately; triolein, trilinolenin, diolein, dipalmitin, maize mono-galactosyl diglyceride, the acetone-chloroplast-filtrate obtained while making the chloroplast acetone powder preparation, and finally a control with no added lipid. After the incubation was completed, the lipids were extracted and fractionated by silicic acid column chromatography and TLC on silica-gel G and the radioactivity in the fractions determined.

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It was found (Table 4) that there was no incorporation of label into galactolipids with triolein, trilinolenin or dipalmitin as substrates. Incubation with diolein resulted in the incorporation of 23% of the label almost entirely into monogalactosyl diglyceride. With maize monogalactosyl diglyceride 26% of the label was incorporated mainly into digalactosyl diglyceride. Incubation with the acetone extract from the chloroplasts resulted in 18% incorporation 86% of which was found in the mono- and 14% in the di-galactosyl diglyceride. It appears from these results that although diolein was an effective acceptor of the first galactosylation it was ineffective as an acceptor for the second galactosylation. Maize monogalactosyl diglyceride, under the same conditions, was able to accept the second galactosylation. This suggests that there could have been two or more enzyme systems differing in their specificity for different degrees of unsaturation of the fatty acid chains. The inability of the chloroplast filtrate to proceed further than the first galactosylation, suggests that degalactosylation of the galactosyl diglycerides may have occurred during extraction, thus depleting the enzyme of acceptors for the second galactosylation. The monogalactolipid formed from the resulting diglycerides was probably at an unsuitable level of unsaturation for digalactolipid synthesis. It follows from this that it may be necessary for some degree of unsaturation of the fatty acid residues in the monogalactosyl diglycerides to occur before the second galactosylation can take place.

EXPERIMENTAL

Growth of maize seedlings. Seeds of *Zea mays* cv. Golden Bantam were soaked for 24 hr, dipped in 0·166% formalin, washed, sown in plastic trays of vermiculite and incubated in darkness at 30° for 7 days. Half the seedlings were kept in the dark and half removed to a second incubator at 30° which was identical except for the provision of constant illumination from a 40 W annular warm white fluorescent light giving 250 lm/m² at half the height of the leaves. The seedlings were sampled after a further 72 hr darkness (i.e. 10 days in all) and 0, 6, 12, 24, 48 and 72 hr of illumination. The aerial parts were cut off at the seed and the glycolipid and chlorophyll contents of the shoots determined.

Feeding UDP-[^{14}C]-Galactose to maize shoots. Dark-grown (7-day-old) maize shoots were bound in batches of 10 and their cut ends placed in 4 ml of aqueous solution containing 3·2 nM and 0·99 μCi UDP-[^{14}C]-galactose. Batches of shoots were then illuminated as above for 0·5, 1, 3, 6, 12, 24, 48 and 72 hr. The ninth batch was given 48 hr of continuing darkness and the tenth batch 24 hr of illumination followed by 48 hr of darkness.

Preparation of maize chloroplast acetone powder and incubation with substrates. Chloroplasts were isolated from the shoots (100 g) of 14-day-old maize seedlings grown in continuous illumination of 2000 lm/m². An acetone powder was prepared from the centrifuge pellet using the method of Ongun and Mudd.[14] A yield of 237 mg of powder was obtained and stored in a desiccator at 4°. The acetone filtrate was evaporated to dryness under N_2 in rotary evaporator, resuspended in 10 ml $CHCl_3$ and stored under N_2 at −20°. The various incubation substrates were dissolved in 0·2 ml of acetone and mixed with the chloroplas acetone powder by shaking for 30 sec. The acetone was then driven off using a stream of N_2 and the dry residue mixed with 2 ml of 0·1 M Tris buffer of pH 7·4. A UDP-galactose solution of 10 nm in the same Tris buffer was activated by adding 0·8 nM, 0·25 μCi of UDP-[^{14}C]-galactose. The incubations were carried out by mixing 0·5 ml of chloroplast powder-substrate suspension with 1 ml of labelled UDP-galactose and continuously illumina ting and shaking at 37° for 1 hr.

Chlorophyll extraction and estimation. Samples of 10 g fr. wt were homogenized and extracted with a total of 60 ml of Me_2CO at 4°. This extract was filtered on a sintered glass funnel and adjusted to 100 ml of 80% Me_2CO solution. Chlorophyll concentration[20] = 2·78 A.

Extraction and purification of the total lipid fraction. The plant material was homogenized and extracted with 50 ml $CHCl_3$–MeOH (2:1) per g fr. wt,[21,22] This crude extract was dried in a rotary evaporator, extracted once more and concentrated to 5 ml. All these operations were carried out under N_2. A suspension of G25 coarse Sephadex in 50% aq. MeOH was used to make a column bed 10 × 2·54 cm. This was washed in

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the series of $CHCl_3$–MeOH–HOAc–H_2O mixtures detailed by Siakotos and Rouser.[23] The 5 ml of total lipid extract was then washed through the column using a mixture of $CHCl_3$–MeOH (19:1) saturated with H_2O. The eluted lipid was finally dried to constant weight in a vacuum desiccator flushed with N_2. A 5 × 2·54 cm column bed was prepared from a slurry of Unisil (100–200 mesh) in $CHCl_3$.[24] The total lipid extract was dissolved in $CHCl_3$ and applied to the column. Neutral lipids were then eluted with 175 ml $CHCl_3$, glycolipids and sulpholipids with 700 ml Me_2CO and the remaining lipids with 175 ml MeOH.[24]

Preparative TLC of the glycolipid fractions. The glycolipid fraction was concentrated and streaked onto TLC plates spread with a layer of Silica-gel G (Merck) 500 μm thick and activated at 110° for 1 hr. The plates were then developed by a mixture of $CHCl_3$–MeOH–OHAc (65:25:8) and the resulting bands rendered visible under UV by spraying with 0·0012% aq. Rhodamine 6G.[25] The relevant bands were then scraped off the plate and eluted in Me_2CO.[26] Radioactive samples were transferred to planchettes, dried and counted using a Panax solid scintillation counter.

Methanolysis of the glycolipids. Up to 5 mg of glycolipid and 4 ml of anh. 0·5 N methanolic-HCl were placed in a strong glass phial. This was closed by a glass stopper fitted with a Teflon sleeve and held in place by springs. The phial was then incubated at 75° for 24 hr and cooled. A known quantity of D-mannitol dissolved in MeOH was added at this stage as an internal standard. The methyl fatty acids liberated by the methanolysis were then extracted using a total of 6 ml of hexane and the methyl glycosides remaining in the MeOH evaporated to dryness on an H_2O bath under N_2.

Gas chromatography of the glycolipid sugars. The methyl glycosides were dissolved in 1 ml of anh. pyridine to which 0·2 ml hexamethyldisilazane and 0·1 ml trimethylchlorosilane were added to form their trimethylsilyl derivatives.[27] The pyridine was evaporated off under N_2 and the silanized sugars redissolved in CS_2.[28] This avoids the large pyridine peak on the gas chromatogram. The silanized sugars were separated by GLC using an FID and a 2 m 0·125 in o.d., 2·5% OV-1 column with an injection port temp. 255°, the column temp. 150° and a N_2 flow rate of 32 ml/min.

Gas chromatography of the glycolipid fatty acids. The methyl fatty acids dissolved in hexane were concentrated and separated before and after dehydrogenation,[29] by GLC fitted with an FID and *either* a 2 m 0·125 in o.d. diethyleneglycol succinate column, N_2 flow of 15 ml/min, injection port temp. 280° and column—temp. 181° *or* a 10% Apiezon L column at 197° with N_2 flow of 28 ml/min.

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