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The Lipid Composition of a Barley Mutant Lacking Chlorophyll *b*

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The acyl-lipid composition of a barley mutant that contained no detectable chlorophyll *b* was studied. This mutant contained chloroplasts that were much less organized than chloroplasts of normal barley. The mutant contained all the normal acyl lipids, with small increases in the relative concentration of phosphatidylglycerol and diacylsulphoquinovosylglycerol compared with other acyl lipids. The fatty acid composition of the galactosylglycerides was unchanged, but most other lipids of the mutant barley contained lower amounts of α-linolenic acid compared with normal. There was no difference in the *trans*-hexadec-3-enoic acid content of phosphatidylglycerol, which was evidence against this lipid being involved in grana stacking.

Acyl lipids are major constituents of chloroplasts. Of the various lipid types, the three glycolipids, diacylgalactosylglycerol (monogalactosyldiglyceride) diacylgalabiosylglycerol (digalactosyldiglyceride) and diacylsulphoquinovosylglycerol (plant sulpholipid) and two phospholipids, phosphatidylglycerol and phosphatidylcholine, are important (Harwood, 1977*a*). In spite of their widespread occurrence no specific function has been proved for any individual acyl lipid. Attempts to define a role for these compounds in chloroplasts have included the use of lipolytic enzymes (Shaw *et al.*, 1976), solvents (Krupa & Baszynski, 1975) and antisera (Menke *et al.*, 1976), together with a simultaneous analysis of metabolic or structural characteristics. As a result of these types of experiments, some generalized functions have been suggested (cf. Harwood, 1977*a*). Unfortunately, these kinds of treatments suffer from the disadvantage that it is difficult to affect specific lipid molecules or regions of the chloroplast. Accordingly, for a number of years biochemists have analysed leaf tissue or chloroplasts from a wide variety of photosynthetic organisms. Higher-plant chloroplasts are remarkably similar in structure and lipid composition, so that this approach has not been particularly fruitful. Where significant differences occur, they do so between species that are quite distinct (e.g. blue–green algae and higher plants), so that they may merely reflect metabolic differences (Hitchcock & Nichols, 1971). However, Tuquet *et al.* (1977), in following up the work of Bishop *et al.* (1971), have examined the lipids in chloroplasts isolated from maize bundle-sheath and mesophyll cells. The only noticeable difference that they found was in the distribution of phosphatidylglycerol containing *trans*-hexadec-3-enoic acid, and they concluded that this molecule may be involved in the formation of grana stacks.

Another method of study has been to examine the lipid contents of leaves, or plastids isolated therefrom, during development. The experiments have included changes brought about by the greening of etiolated tissue (e.g. Appelqvist *et al.*, 1968; Sellden & Selstam, 1976) or its exposure to light of specific wavelengths (Tevini, 1977) or by analysing serial sections of the leaves of monocotyledons (Bolton & Harwood, 1977*b*; Leech *et al.*, 1973; Leese & Leech, 1976).

The most specific approach to finding a role for acyl lipids in chloroplasts is the examination of deficient mutants. This method has, of course, been used with considerable success in the microbial field, where viable mutations can be easily induced, but very few suitable plant mutants exist. Sinensky (1977) examined a *Chlorella* mutant lacking diacylsulphoquinovosylglycerol and concluded that previous suggested roles for this lipid in binding and orientating chlorophyll (Rosenberg & Pecker, 1964) were not correct. Instead they suggested that diacylsulphoquinovosylglycerol may be involved in the activity of phosphoribulokinase. Viable mutants lacking chlorophyll *b* have been found in a number of plants, but only in barley have high photosynthetic rates been reported (Highkin & Frenkel, 1962). Because this mutant appears to have impaired grana formation (Goodchild *et al.*, 1966) and lipids have been suggested possibly to be involved in this process (Kreutz, 1969; Leese & Leech, 1976; Tuquet *et al.* 1977) we have compared the lipid composition of the chlorophyll *b*-lacking mutant with that of normal barley.

Experimental

Materials

Fatty acid standards, phosphatidylcholine and phosphatidylethanolamine were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K., and phosphatidylinositol was from Koch–Light Laboratories, Colnbrook,

Bucks. SL3 0BZ, U.K. Phosphatidic acid was prepared from phosphatidylcholine by the action of phospholipase D [Boehringer Corp (London), Lewes, Sussex BN7 1LG, U.K.] (Kates & Sastry, 1969). Phosphatidylglycerol was prepared from *Vicia faba* leaves, and diacylgalactosylglycerol, diacylgalabiosylglycerol and diacylsulphoquinovosylglycerol were from Swiss chard (*Beta vulgaris* cultivar Cicla) leaves (O'Brien & Benson, 1964). The purity of all standards was checked by g.l.c. and t.l.c. and, where necessary, they were purified to homogeneity by t.l.c. Solvents were obtained from Koch–Light, and re-distilled as previously described (Bolton & Harwood, 1977*a*). Butylated hydroxytoluene was from BDH Chemicals, Poole, Dorset, U.K.

Plants

Barley seeds were germinated in John Innes seed compost at 22°C in an illuminated growth cabinet by using a 16h light/8h dark cycle. Illumination was 100 (photosynthetically active wavelengths) $\mu E/m^2$ per s.

Lipid extraction and separation

Lipids were extracted from plant material as previously described (Heinz & Harwood, 1977) and the lipids separated either by ion exchange chromatography on DEAE-cellulose DE-23 (Whatman Biochemicals, Maidstone, Kent, U.K.) followed by t.l.c. on silica gel G (E. Merck, Darmstadt, Germany) with chloroform/methanol/acetic acid/water (170:30:20:7, by vol.) as solvent or by t.l.c. on silica gel G with acetone/benzene/water (91:30:8, by vol.) as solvent (Heinz & Harwood, 1977). The positions of the lipid bands were revealed by spraying the plates with aq. 0.001% (w/v) Rhodamine 6G and their identities deduced by comparison with authentic markers. Confirmation of the identities was provided, with normal barley, by degradation of the complex lipids as previously described (Bolton & Harwood, 1977*a*). Solutions contained 0.1% (w/v) butylated hydroxytoluene to prevent oxidation.

The fatty acids of the complex lipids were transesterified, and non-esterified fatty acids esterified, with 2.5% (v/v) H_2SO_4 in methanol at 65°C for 2h. Previous experiments had shown these conditions to result in quantitative formation of methyl esters of all lipids concerned. The fatty acyl esters were separated in glass columns (2.5m × 0.7mm) of 15% EGSS-X on Supelcoport (100–120 mesh; Supelco, Bellefonte, PA, U.S.A.) at 190°C by using a Perkin–Elmer F33 gas chromatogram fitted with a Varian CDS-111 integrator.

The amounts of fatty acids were calculated by inclusion of a methyl pentadecanoate standard. Quantification of the acyl lipids was made from the fatty acid data.

Chloroplast purification

Chloroplasts were purified by the method of Leese & Leech (1976).

Chlorophyll determination

Chlorophyll was determined by the method of Bruinsma (1961) and the spectral characteristics were studied by the procedure of Boardman & Highkin (1966).

Electron microscopy

Pieces of tissue were cut from the central portion of the second leaf of mutant or normal barley plants and fixed in glutaraldehyde, post-fixed in OsO_4 and double-stained with uranyl acetate/lead citrate. They were viewed with an AE1-EM6 electron microscope.

Results and Discussion

The mutant barley was analysed for chlorophyll *a* and *b* contents and, in agreement with Boardman & Highkin (1966), contained a ratio of chlorophyll *a*/*b* over 500.

Representative electron micrographs of leaf tissue obtained from normal and mutant barley plants were then examined to ascertain the relative morphology of their chloroplasts. As Goodchild *et al.* (1966) first observed for mutant chlorophyll *b*-lacking barley, the lamellar structure of the chloroplast appeared to be disorganized in comparison with normal plants. Chloroplasts from both leaf types were compared by a number of criteria as shown in Table 1. All five comparisons showed highly significant differences between the chloroplasts of normal and those of the chlorophyll *b*-lacking mutant barley. In particular, the halving of the amount of stacked lamellae per section and of the number of lamellae per granum in the mutant emphasized the lack of organization. These values compare very well with those obtained for chlorophyll *b*-lacking barley by Goodchild *et al.* (1966).

After establishing that the barley species used exhibited markedly different chloroplast organization, we analysed the complex lipid contents of their respective leaves. The total acyl-lipid and fatty acid content of the leaves are shown in Table 2. The leaves from normal barley plants contained significantly more lipid on a wet-weight basis than the chlorophyll *b*-lacking mutant of the same age. However, the rate of growth of the normal barley was faster than for the mutant, and for leaves of approximately the same size (8 days for normal plants, 14 days for chlorophyll *b*-lacking plants) the total lipid contents were comparable. The total fatty acid analysis revealed a higher content of linolenic acid in normal plants than in mutant barley of a comparable age. The mutant

Table 1. *Characteristics of chloroplast sections from normal and mutant barley leaves*
For the method of analysis see the Experimental section. Results are expressed as means ± S.D. ($n = 6$). Significance was analysed by Student's *t* test.

	10^{-4} × Length of stacked lamellae/chloroplast section (m)	10^{-4} × Length of unstacked lamellae/chloroplast section (m)	Grana/chloroplast section	Lamellae/granum	Grana with eight or more lamellae/chloroplast section
Normal	1.07 ± 0.12	0.21 ± 0.02	42.6 ± 4.3	5.6 ± 0.3	6.5 ± 0.8
Mutant	0.43 ± 0.02	0.33 ± 0.02	29.8 ± 0.6	2.8 ± 0.2	2.2 ± 0.4
Significance (*P*)	<0.001	<0.05	<0.001	<0.001	<0.005

Table 2. *Total acyl lipid and fatty acid content of normal and chlorophyll b-lacking mutant barley leaves*
For details see the Experimental section. Means ± S.D. are shown (where appropriate) for the numbers of analyses shown. Abbreviation: tr., <0.05.

Plant	Age (days)	Total acyl lipid (μg/mg wet wt.)	No. of analyses	Fatty acid composition (% of total) $C_{16:0}$	$C_{16:1}$	$C_{16:1(t-3)}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Normal	8	10.9 ± 0.6	3	13.2 ± 0.2	3.0 ± 0.3	2.5 ± 0.2	2.1 ± 0.3	6.8 ± 1.3	6.7 ± 0.2	64.0 ± 3.7
	14	14.3 ± 0.4	2	13.4 ± 0.2	1.1 ± tr.	2.5 ± 0.2	1.6 ± 0.1	3.5 ± 0.2	8.9 ± 0.2	68.9 ± 0.2
Mutant	8	7.5 ± 0.8	4	18.2 ± 0.4	2.2 ± 0.2	3.1 ± 0.4	2.5 ± 0.3	7.1 ± 0.4	9.8 ± 0.4	57.1 ± 2.3
	14	10.5 ± 0.4	2	15.5 ± 1.7	2.2 ± 0.4	3.0 ± 0.5	2.9 ± 0.4	6.9 ± 0.8	8.7 ± 1.0	60.8 ± 4.2

Table 3. *Acyl-lipid analysis of leaves of normal and chlorophyll b-lacking barley*
For details of analysis see the Experimental section. Means ± S.D. are shown ($n = 3$). Other acyl lipids consisted mainly of phosphatidylinositol. Leaves were from 11–12-day-old plants.

Acyl lipid Source ...	Proportion of lipid (g/100g total) Normal	Mutant
Triacylglycerol and non-esterified fatty acid	3.6 ± 0.9	3.6 ± 0.3
Diacylgalactosylglycerol	40.0 ± 3.1	37.0 ± 0.7
Diacylgalabiosylglycerol	23.7 ± 0.7	23.8 ± 0.2
Diacylsulphoquinovosyl-glycerol	3.7 ± 0.7	5.3 ± 0.9
Phosphatidylcholine	12.5 ± 1.1	12.8 ± 0.9
Phosphatidylethanolamine	3.7 ± 0.1	4.3 ± 0.6
Phosphatidylglycerol	8.1 ± 0.6	9.6 ± 0.9
Phosphatidic acid and cardiolipin	3.6 ± 0.1	2.7 ± 0.4
Others	1.1 ± 0.2	0.9 ± 0.1

barley had a higher proportion of palmitic acid particularly after 8 days of growth, but there was no significant difference in the amount of *trans*-hexadec-3-enoic acid compared with normal barley. The overall fatty acid analysis of normal barley was similar to previous data (Hitchcock & Nichols, 1971; Tevini, 1977).

The complex-lipid contents of the two varieties of barley are shown in Table 3. In both leaves the characteristically high content of diacylgalactosylglycerol and diacylgalabiosylglycerol was observed. A small increase in the relative percentage of two acidic lipids, diacylsulphoquinovosylglycerol and phosphatidylglycerol, was observed in the leaves of the mutant barley. This may reflect the increase in the ratio of stromal to granal lamellae in the mutant, since stromal lamellae have been found to be richer in these two lipids (Bahl *et al.*, 1976; Douce *et al.*, 1973). The overall patterns were similar to data for barley (Tevini, 1977) and other higher plants (Hitchcock & Nichols, 1971). The fatty acids of isolated lipids (Table 4) showed more differences. Notably the linolenic acid content of diacylsulphoquinovosylglycerol and the phospholipids in the leaves from chlorophyll *b*-lacking barley was lower than in the normal variety. This decrease was accompanied by an increase in linoleic acid and oleic acid. In contrast, the two lipids richest in linolenic acid showed either no difference between the two barleys (diacylgalabiosylglycerol), or a small increase (diacylgalactosylglycerol) in the mutant tissue. These differences account for the overall patterns of fatty acids in the two barleys as shown in Table 2. It is noteworthy that although the phosphatidylglycerol of the leaves from chlorophyll *b*-lacking barley had a lower linolenic acid content than normal leaves, the proportion of *trans*-hexadec-3-enoic acid was unchanged. The data for normal barley are comparable with results

Table 4. *Fatty acid composition of individual leaf lipids*
For details of analysis see the Experimental section. Abbreviations: tr., <0.1; n.d., not detected. Results are the average of three experiments. Leaves were from 11–12-day-old plants.

Lipid	Source	Fatty acid composition (% of total)							
		$C_{16:0}$	$C_{16:1}$	$C_{16:1(t\text{-}3)}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Others
Diacylgalactosylglycerol	Normal	9.5	1.7	n.d.	0.8	0.6	2.2	85.2	tr.
	Mutant	3.1	tr.	n.d.	0.7	1.6	3.4	90.8	0.4
Diacylgalabiosylglycerol	Normal	14.8	2.4	n.d.	2.0	1.7	4.1	75.0	tr.
	Mutant	18.9	tr.	n.d.	1.8	1.4	3.5	73.6	0.8
Diacylsulphoquinovosyl-glycerol	Normal	32.3	2.7	n.d.	1.3	2.2	4.7	54.5	2.3
	Mutant	29.2	4.3	n.d.	3.1	5.2	20.5	35.7	2.0
Phosphatidylcholine	Normal	30.2	2.3	n.d.	2.9	5.1	25.1	31.5	2.9
	Mutant	25.4	tr.	n.d.	1.6	10.1	36.5	25.9	0.5
Phosphatidylglycerol	Normal	18.0	1.0	26.9	2.6	2.1	11.2	37.6	0.6
	Mutant	18.5	3.6	25.7	1.6	6.7	13.5	29.5	0.9
Phosphatidylethanolamine	Normal	17.2	3.7	n.d.	0.8	2.1	25.7	50.4	0.1
	Mutant	18.3	1.9	n.d.	0.3	7.4	32.3	39.8	tr.
Phosphatidylinositol	Normal	24.3	4.0	n.d.	10.6	13.9	11.1	29.1	7.0
	Mutant	28.7	2.8	n.d.	11.3	11.2	27.5	11.3	7.2

Table 5. *Comparison of the lipids of chloroplasts isolated from normal and chlorophyll b-lacking mutant barley leaves*
Chloroplasts were isolated and analysed as described in the Experimental Section. Leaves were from 11–12-day-old plants.

Source	Lipid	Proportion of total acyl lipids (%)	Fatty acid composition (% of total)							
			$C_{16:0}$	$C_{16:1}$	$C_{16:1(t\text{-}3)}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Others
Normal	Diacylgalactosylglycerol	52.0	3.2	0.2	n.d.	tr.	0.7	4.1	91.6	0.2
	Diacylgalabiosylglycerol	27.5	15.1	0.5	n.d.	1.3	1.7	4.7	75.7	1.0
	Diacylsulphoquinovosyl-glycerol	6.2	28.4	4.8	n.d.	4.4	5.4	7.6	48.1	1.3
	Phosphatidylcholine	4.3	30.4	5.0	n.d.	5.1	9.2	15.9	33.6	0.8
	Phosphatidylglycerol	7.4	14.2	tr.	31.0	3.2	5.2	5.3	40.0	1.1
Mutant	Diacylgalactosylglycerol	51.3	2.3	0.2	n.d.	1.3	3.2	4.0	89.0	tr.
	Diacylgalabiosylglycerol	18.5	12.1	1.1	n.d.	1.1	1.9	7.0	76.8	tr.
	Diacylsulphoquinovosyl-glycerol	11.1	27.5	3.9	n.d.	2.0	5.1	21.1	38.5	1.9
	Phosphatidylcholine	7.7	26.7	3.0	n.d.	4.2	8.5	27.4	28.2	1.0
	Phosphatidylglycerol	12.0	15.1	0.4	32.1	3.2	4.7	5.2	38.4	0.9

obtained for some of the lipids shown in Table 4 by Tevini (1977). The α-linolenic acid content of the lipids was, however, noticeably higher than for greening etiolated barley (Appelqvist *et al.*, 1968; Sellden & Selstam, 1976). The increase in total phosphatidylglycerol in the leaves from mutant barley (Table 3) without any change in its *trans*-hexadec-3-enoic content (Table 4) fully accounted for the small rise in the proportion of this acid in chlorophyll *b*-lacking barley (Table 2).

The differences in the fatty acid composition of complex lipids were also observed when chloroplasts were isolated and analysed (Table 5). It has often been noted that higher-plant chloroplasts, including those from barley (Tevini, 1977), contain large amounts of three glycolipids, diacylgalactosylglycerol, diacylgalabiosylglycerol and diacylsulphoquinovosylglycerol. In addition, the characteristic phospholipid is phosphatidylglycerol, which is present in excess of phosphatidylcholine (cf. Hitchcock & Nichols, 1971; Harwood, 1977*b*; Mazliak, 1977). Some evidence indicates that these lipids are not evenly distributed within the lamellar membranes (Allen *et al.*, 1966, 1972; Bahl *et al.*, 1976) and it is clear that the chloroplast envelope has a different composition to the lamellae (e.g. Mackender & Leech, 1974).

Two interesting differences can be noted between the analyses for chloroplasts from normal and mutant barley (Table 5) and they complement the data for whole leaves. First, the two acidic lipids, phosphatidylglycerol and diacylsulphoquinovosylglycerol, were both increased as a percentage of the total acyl lipids in mutant barley. Secondly, linolenic acid was decreased in three of the lipids, but interestingly, not in the major linolenic acid-containing lipids, the acylgalactosylglycerols. Moreover, the *trans*-hexadec-3-enoic acid content of phosphatidylglycerol was the same in chloroplasts isolated from either barley variety. The suggestion by Tuquet *et al.* (1977) that this particular fatty acid functions by aiding grana structure does not seem likely, at least in barley. Thus a halving of the grana stacking (Table 1) is accompanied by a net increase in the percentage of *trans*-hexadec-3-enoate present (Table 2) and by no change in its relative concentration in phosphatidylglycerol (Tables 4 and 5). It appears likely therefore, that the differences in the content of this acid in mesophyll compared with the bundle-sheath cells of maize (Tuquet *et al.*, 1977) do not simply reflect differences in grana stacking. Whether or not phosphatidylglycerol containing *trans*-hexadec-3-enoic acid could act as a fixed boundary lipid (Anderson, 1975; Leese & Leech, 1976) would depend on the numbers of chlorophyll molecules that are associated together in stromal and in grana lamellae. There is no evidence at present for such an idea.

It is noteworthy that phosphatidylglycerol and diacylsulphoquinovosylglycerol were present as higher percentages of the total acyl lipids of chloroplasts from mutant barley. This agreed with the higher proportions of these lipids in relatively undifferentiated maize (Leech *et al.*, 1973; Leese & Leech, 1976) or barley (P. Bolton & J. L. Harwood, unpublished work) plastids. In addition, data from chloroplasts that had been subfractionated also indicated an increased concentration of phosphatidylglycerol and diacylsulphoquinovosylglycerol in the stromal lamellae fractions (Allen *et al.*, 1966, 1972; Bahl *et al.*, 1976). The relatively disorganized chloroplasts of chlorophyll *b*-lacking barley do differ from the partly differentiated chloroplasts present in normally developing tissue (Leech *et al.*, 1973; P. Bolton & J. L. Harwood, unpublished work) in that the linolenic acid content of the acylgalactosylglycerols was as high as in differentiated chloroplasts (Tables 4 and 5).

In conclusion, we consider that the data in the present paper make a specific role for the acyl lipids in the morphological appearance of the barley chloroplast unlikely. Swanson *et al.* (1973) reached a similar conclusion after experiments on the electron-microscopic appearance of solvent-extracted plant membranes. In fact, it seems possible that if a specific role is found it will relate to metabolism (Menke *et al.*, 1976; Harwood, 1977*a*; Sinensky, 1977) rather than to a function in grana stacking.

The chlorophyll *b*-lacking mutant barley (cultivar Chlorina 2) seeds were a gift from Professor R. J. Ellis, Department of Biological Science, University of Warwick. Phosphatidylglycerol was prepared by Mr. M. Percival and glycolipids by Mr. D. D. Burns of this department. Electron microscopy was carried out by Mrs. C. Winters, Department of Zoology, University College, Cardiff.

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