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GREENING-RELATED LIPID CHANGES IN LEAVES, PROTOPLASTS AND A PLASMAMEMBRANE-ENRICHED FRACTION OF PEA

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Key Word Index—*Pisum sativum*; Leguminosae; pea; light; membrane lipids; protoplasts; plasmamembrane-enriched fraction.

Abstract—Light-induced changes in the membrane lipid compositions were studied in pea leaves and in protoplasts and a plasmamembrane-enriched fraction (PMEF)* of pea leaves. PC, PE, PI, PG, PA, MGDG, DGDG and SL were identified as the glycerolipids. The relative levels of various membrane lipids changed due to light-induced greening. There was an increase in the galactolipids of leaves and leaf protoplasts. The galactolipid constituent of the PMEF was very low and showed no change. Among the plasmamembrane phospholipids, PI increased with a concomitant decrease in PC.

INTRODUCTION

Light-induced changes in the membrane lipids of leaves have been shown to take place mainly in the galactolipids [1–3], which are the major lipids of chloroplasts [4, 5]. Such changes in chloroplast lipids are correlated to the synthesis of grana membrane [2, 3, 6, 7]. Although the lipid compositions of isolated chloroplasts and different membrane fractions of chloroplasts are known [7–10], the status of plasmamembrane lipid composition during this stage of development is not well understood. In the present study, light-induced changes in membrane lipids were investigated. The results demonstrate that the majority of plasmamembrane lipids are insensitive to light and the lipid changes observed in leaves and leaf protoplasts are mainly due to the synthesis of grana membrane.

RESULTS AND DISCUSSION

Lipid composition of leaves and leaf-protoplasts

Light-induced changes in the lipid compositions of leaves and leaf protoplasts were investigated to find out whether the lipid composition of leaf protoplasts was comparable to that of leaves. Leaves of light-grown plants contained *ca* 44% more total lipids than the dark control, on a mg protein basis (Table 1), which was mainly due to an increase in galactolipids. Tremolieres and Lepage [1] correlated the greening-related increase in membrane lipid content to the development of chloroplasts and it was shown that the galactolipids were the major lipids of the chloroplast membrane [4, 5]. As a consequence of the increase in galactolipids, the relative levels of phospholipids and neutral lipids, which were *ca* 57 and 21% of the total lipids, respectively, decreased during greening (Table 1).

Among the galactolipids, the relative level of MGDG showed an *ca* 80% increase in leaves of light-grown plants, which also resulted in an increase in the ratio of MGDG/DGDG from 1.4 to 2.0 (Table 2). Similar results were reported by Bahl *et al.* [7], who analysed the lipid compositions of membrane fractions from etioplasts and chloroplasts and showed that DGDG and MGDG were predominant in the envelope and grana membrane, respectively. During greening, there was a net synthesis of grana membrane in the pre-existing proplastids of dark-grown leaves, leading to an increase in the relative level of MGDG [2, 6, 7]. Therefore, the changes observed during greening of pea leaves were probably due to the net synthesis of chloroplast membrane.

The lipid changes observed in leaf protoplasts were quite comparable to those of leaves (Tables 1 and 2). However, there was a drop in the relative level of neutral lipids of protoplasts as compared to that of leaves. This could be due to the removal of extra-membrane lipids during protoplast preparation. Thus Yamada *et al.* [11] reported that phospholipases might be activated during the isolation of protoplasts. Higher levels of PA and lyso derivatives of cellular phospholipids have been shown to indicate such degradative changes [11–13]. Since the relative levels of PA and other phospholipids of protoplasts were similar to those of leaves, it is clear that the leaf protoplasts prepared in the present study were free of any degradative change.

Qualitatively, the leaves and leaf protoplasts of both dark- and light-grown pea plants had similar compositions of membrane lipids with PC > PE > PI > PG > PA among phospholipids and MGDG > DGDG > SL among galactolipids (Table 2). Thus, as reported earlier [1, 2], the greening-related changes in membrane lipids were found to be only quantitative in nature.

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Abbreviations: DGDG, digalactosyl diglyceride; MGDG, monogalactosyl diglyceride; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PMEF, plasmamembrane-enriched fraction.

Table 1. Light-induced changes in the total lipid composition

Experimental material	Treatment*	Total lipids (mg/mg protein)	Amount (% total) Phospho-lipids	Galacto-lipids	Neutral lipids
Leaves	Dark	0.55	57	21	23
	Light	0.79	50	33	17
Leaf protoplasts†	Dark	0.51	60	23	16
	Light	0.74	52	35	13
Plasmamembrane-enriched fraction	Dark	0.90	78	6	16
	Light	0.91	77	7	16

*Dark, 16 days dark-grown, light, 12 days dark + 4 days light.
†1 mg protoplast protein accounted for *ca* 1.25×10^6 protoplasts.
Results are the mean of three experiments.

Table 2. Light-induced changes in the phospholipid and galactolipid composition

Experimental material	Treatment*	Phospholipids + galactolipids (μmol/mg protein)	% Phospholipids + galactolipids PC	PE	PI	PG	PA	MGDG	DGDG	SL
Leaves	Dark	0.57	33	20	9	7	3	14	10	4
	Light	0.81	18	16	12	9	4	25	12	4
Leaf protoplasts	Dark	0.54	34	22	8	7	4	13	8	4
	Light	0.80	21	19	10	10	4	21	10	5
Plasmamembrane-enriched fraction	Dark	0.97	44	27	14	4	5	2	2	2
	Light	0.95	37	29	19	3	6	2	2	2

*Dark, 16 days dark-grown; light, 12 days dark + 4 days light.
Results are the mean of three experiments.

Lipid composition of plasmamembrane-enriched fraction (PMEF)

The PMEF of pea leaves had a lipid : protein ratio of 0.9 (Table 1) which was comparable to that of corn roots [13]. The membrane lipid composition of PMEF was qualitatively the same as that of leaves and leaf-protoplasts (Table 2). PC > PE > PI > PG > PA comprised the phospholipids and accounted for *ca* 78% of total plasmamembrane lipids. As in leaves and protoplasts, PC and PE were the major constituents of the PMEF, accounting for *ca* 71% of the glycerolipids. The relative level of PI was higher than that of leaves and leaf-protoplasts. Galactolipids were very low and never accounted for more than *ca* 6% of the total plasmamembrane lipids. Whereas galactolipids have been shown to be present in the plasmamembrane of many systems [13–16], their content was noted to be only minimal [13, 16].

Our results demonstrate that, in contrast to leaves and leaf protoplasts, there was no significant light-induced change in the amount of total plasmamembrane lipids (Table 1). The greening-related increase in the MGDG/DGDG ratio of leaves and leaf protoplasts was also not observed in the PMEF (Table 2). However, a higher proportion of PI in the PMEF and its further increase during greening with a concomitant decrease in PC may be of physiological significance. Cocucci and Ballarin-Denti [17] recently reported a significant change in the composition of plasmamembrane phospholipids of germinating radish seeds which had a regulatory effect on the plasmamembrane-bound ATPase activity. It would be interesting to ascertain the significance of the change in the relative levels of PI and PC of plasmamembrane phospholipids during greening of pea leaves.

EXPERIMENTAL

Chemicals. Macerozyme and cellulase for protoplast isolation were from Onozuka Enzyme Products, Kinki Yakult Mfg. Co., Shingikancho, Nishinomiya, Japan.

Plant material and growth conditions. Pea (*Pisum sativum* L. var. Bonneville) seeds were soaked in H_2O for 24 hr and grown on moist cotton pads. Plants were maintained in a dark room (with only a green safe light) at 26°. To induce greening, light treatment was given to 12-day-old dark-grown plants from a fluorescent light source (intensity 1200 $\mu W/cm^2$ at plant level) for 4 days. Control plants were kept in the dark for 16 days.

Preparation of protoplasts. Leaf tissue slices were incubated for 4 hr in 1.5% macerozyme and 3% cellulase in 0.6 M mannitol (pH 5.6) at 26°. Later, the incubation mixture was filtered through a nylon mesh (100 μm) and the protoplasts in the filtrate were collected by centrifugation at 150 *g* for 3 min. Protoplasts were washed twice with mannitol (0.6 M) and further purified by layering on 5% Ficoll-400 in 0.6 M mannitol. The intact proto-

plasts were collected as a pellet by centrifugation at 1000 *g* for 5 min.

Preparation of the PMEF. The PMEF was prepared from leaf protoplasts essentially according to the procedure of ref. [18], with a modification to inhibit phospholipase D and phosphatidic acid phosphatase activity [12]. Accordingly, choline chloride (4% w/v), glycerophosphate (4% w/v) and ethanolamine (4% v/v) were added to all solns used in the preparation of PMEF.

Lipid extraction. Samples were fixed in 5 vol. of boiling *i*-PrOH (w/v for leaves and v/v for protoplast- and PMEF-suspension) for 5 min prior to lipid extraction. Total lipids were extracted according to the procedure of ref. [19] and the extract was washed with 0.2 vol. 0.9% NaCl to remove non-lipid contaminants [20]. The solvent phases were allowed to separate and the lower ($CHCl_3$) phase containing the total lipids was evapd under reduced temp. and pressure. The conc. lipid was stored under N_2 until required.

Lipid analysis. This was carried out using a two-step chromatographic procedure involving silica gel CC [21] and TLC. The Me_2CO (galactolipid) fraction from CC was concd and subjected to TLC [22] on silica gel-H using $CHCl_3$–MeOH–HOAc–H_2O (170:30:20:7) and the MeOH (phospholipid) fraction was subjected to TLC [23] on $C_2H_2O_4$-impregnated silica gel-H (0.04:1) with $CHCl_3$–MeOH–H_2O (65:25:4).

The spots were visualized by I_2 vapour and their identities established [23, 24]. Determination of total lipids was done gravimetrically and that of phospholipids and galactolipids was accomplished through lipid phosphorus [25] and lipid galactose [22], respectively. The amount of total phospholipids was obtained by multiplying the lipid-phosphorus value by 25 and for total galactolipids the lipid-galactose value by 4.3, 2.6 and 3.4 for MGDG, DGDG and SL, respectively. The lipid content was expressed on a mg protein basis of leaves, protoplasts and PMEF, respectively. Protein was estimated according to the procedure of ref. [26]

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