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LIPID COMPOSITION OF THYLAKOID MEMBRANES FROM LEAVES AND REGREENED SPATHES OF *ZANTEDESCHIA AETHIOPICA*

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Abstract—The glycerolipid and fatty acid composition of thylakoid membranes from two photosynthetic organs, the leaf and regreened spathe, of *Zantedeschia aethiopica* was studied. In regreened spathes, there was a decrease in the monogalactosyl- and digalactosyldiacylglycerols, and an increase in the contents of the sulpholipid, sulphoquinovosyldiacylglycerol and in the phospholipids phosphatidylglycerol and phosphatidylcholine, when compared with those isolated from the leaf. The major difference detected in fatty acid composition of both photosynthetic structures was the absence of the unusual fatty acid, Δ^3-*trans*-hexadecenoic, from phosphatidylglycerol in regreened spathe thylakoid membranes. In spite of these differences, chloroplasts from regreened spathes exhibited a typical organization, with *grana* and stromal regions. The results obtained suggest, that in *Z. aethiopica*, there is no evident correlation between the presence of Δ^3-*trans*-hexadecenoic acid in thylakoid membranes and thylakoid-stacking. The absence of this fatty acid in the regreened spathe is discussed in terms of chloroplast function.

INTRODUCTION

Photosynthetic membranes are composed mainly of lipids and proteins arranged in lipoprotein complexes. The lipid composition of thylakoid membranes of higher plants is unique. Major lipids are the galactolipids, monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) that usually represent more than 75% (mol%) of the total thylakoid lipids. The remainder is mainly composed of the anionic lipids sulphoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) [1]. Phosphaditylcholine (PC) has been reported as a component of the outer envelope membrane [2] and also as a thylakoid component [3]. However, it was shown that thylakoids purified from phospholipase-C-treated intact spinach chloroplasts contained no PC [4], which is a strong evidence that the presence of PC in purified thylakoid membranes is a contaminant from the chloroplast envelope. Another unique feature of these membranes is the high degree of unsaturation due mainly to a very high content of α-linolenic acid (18:3) [5].

PG is the major phospholipid present in thylakoid membranes [6] and is characterized by the presence of the unusual fatty acid Δ^3-*trans*-hexadecenoic acid (16:1-*tr*) esterified specifically to the *sn*-2 position [7]. This fatty acid, which only occurs in thylakoid membranes as a PG component [8], may also be found in other lipid fractions in some seed oils [9]. In etioplasts, C16:1-*tr* is present in relatively low amounts but accumulates upon light-induced chloroplast development, in parallel with the accumulation of LHCII and the development of apressed regions [10, 11]. Although the role of PG-containing 16:1-*tr* in thylakoid membranes has not yet been clarified, it has been implicated in granal stacking [12–15] and in the supramolecular organization of LHCII [16–19]. Nevertheless, the involvement of PG-containing C16:1-*tr* in LHCII oligomer formation and granal stacking has been seriously challenged. 16:1-*tr* is present at normal levels in a mutant of barley with very low amounts of stacking [20] and it was inferred that a mutant of *Arabidopsis* lacking PG-C16:1-*tr* showed no changes either in thylakoid membrane appression or in energy transfer from LHCP to photochemical reaction centres [21, 22].

Calla lily (*Zantedeschia aethiopica*) is a monocotyledon that shows, as a peculiar characteristic, regreening of the fructiferous spathe [23]. This leaf-like structure undergoes a regreening process during its development. The transition from the white stage to a regreened one, is only observed when fructification occurs, otherwise the spathe becomes senescent. Previous work suggested a correlation between cytokinin action and regreening. Endogenous cytokinin-like substances were isolated and identified as 6-*o*-hydroxybenzylaminopurine and its riboside [24, 25]. The application of these hormones or synthetic

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cytokinins to *Z. aethiopica* plants, after inflorescence ablation, induced regreening in a similar way to the natural process [26]. Photosynthetic and photorespiratory rates were also measured [27, 28].

To understand the effects of cytokinins on thylakoid membrane reorganization, the identification and quantification of lipids and fatty acids of thylakoids from leaf and regreened spathes of *Z. aethiopica* was performed and is reported herein.

RESULTS AND DISCUSSION

TLC analysis of total lipids from thylakoid membranes isolated from leaves and from regreened spathes showed the presence of the same lipid classes in both extracts. The spots were identified as MGDG, DGDG, SQDG and the phospholipids, PG and PC, based on their R_fs values and colour reactions with chromogenic reagents. The absence of phosphatidylethanolamine (PE) in both samples was considered to be an indication of non-significative contamination by extra-chloroplastidial membranes [1].

Table 1 shows the results of quantitative analysis of the total lipids from leaf and regreened spathe thylakoid membranes. MGDG and DGDG were the major lipid classes, both comprising more than 70% of the total lipids. SQDG was a minor component of thylakoid membranes from both organs, representing 4–6% of the total lipids. PG and PC together represented less than 20% of the total lipids. These results agree with those usually reported for thylakoids isolated from field-growth plants [6]. In comparison, regreened spathe thylakoid membranes exhibited a slight decrease in MGDG and DGDG and a slight increase in PG levels compared with leaf thylakoids. In these membranes, the amount of SGDG and PC was also higher than in leaf thylakoid membranes. When comparing the total amount of glycolipids and phospholipids of these two samples, thylakoid membranes from regreened spathes exhibited a higher content of glycolipids but a lower content of phospholipids than those in leaf thylakoid membranes.

Table 1. Glycerolipid composition (nmol μmol^{-1} Chl) of thylakoid membranes from leaves and regreened spathes of *Z. aethiopica*

Glycerolipids	Leaf	Regreened spathe
MGDG	1284	1067
mol%	48	43
DGDG	937	799
mol%	35	33
SQDG	115	157
mol%	4	6
PG	215	240
mol%	8	10
PC	131	191
mol%	5	8
Total lipids	2682	2455

In Table 2, the fatty acid composition of each glycerolipid class is summarised. In both samples, MGDG and DGDG contained mainly unsaturated fatty acids, linolenic acid 18:3 being the main one, whose concentration was slightly higher in leaf thylakoids. DGDG had a lower content of 18:3, but a higher content in other fatty acids, specially 18:2 and 16:0. In PG and PC, the content of unsaturated fatty acids was lower than in galactolipids, mostly due to the low content of 18:3 and a high 16:0 content. 16:1-*tr* was present only in PG and was only detected in leaf thylakoids, representing in these extracts *ca* 30 mol% of the total fatty acid composition of PG, being the second most abundant acid. SQDG showed higher amounts of 18:3 in regreened spathe thylakoids than in those from leaves.

Fatty acid composition of the total glycerolipids from thylakoid membranes from both photosynthetic organs studied was dominated by unsaturated fatty acids (Table 2). 18:3 was the major fatty acid present, representing 75% of the total fatty acid content, followed by 16:0 and 18:2. Monounsaturated fatty acids were represented by 18:1, palmitoleic acid 16:1-*c* and 16:1-*tr*, the latter being present only in leaf thylakoid membranes. Only traces of 14:0 were detected in both samples. Regreened spathe thylakoid membranes showed higher contents of all fatty acids identified with the exception of 18:3. The decrease of 18:3 followed increases of other C_{18} fatty acids, namely, 18:0, 18:1 and 18:2. The absence of 16:1-*tr* and the high content of 16:0 in PG of thylakoid membranes from *Z. aethiopica* regreened spathes is similar to that found in a mutant of *Arabidopsis thaliana* lacking 16:1-*tr* [22]. The major difference between the two *Z. aethiopica* samples is the absence of 16:1-*tr* in the regreened spathe thylakoid membranes. The 18:3/18:2 ratio (16.0 in leaf; 9.7 in regreened spathe) and the average number of double bonds (4.9 in leaf; 4.6 in regreened spathe) in the total lipids of thylakoid membranes from the two organs studied revealed a higher degree of unsaturation in leaf thylakoid membranes than in regreened spathe thylakoid membranes.

The apparently ubiquitous occurrence of 16:1-*tr* in thylakoid membranes from higher plant chloroplasts and green algae [29] has led to considerable speculation on its possible role in thylakoid membrane organization or function [8]. The earliest suggestions for a role of PG (16:1-*tr*) in granal stacking were based on the correlation between the level of this acid in PG and the extent of granal stacking during the greening process of etiolated leaves [30, 31]. In addition, several authors presented *in vitro* data, which indicated that the 16:1-*tr* content of PG regulates the supramolecular organization of LHCII and granal stacking [8, 15]. In our work, no differences were observed in thylakoidal organization of chloroplasts from leaves and regreened spathes of *Z. aethiopica*. Ultrastructural observations of palisade cells from regreened spathes showed well-structured chloroplasts, with very well-organized grana regions, similar to those of leaf chloroplasts (Figs 1 and 2). According to our results, no such correlation seems to exist between granal stacking and the presence of 16:1-*tr* in *Z. aethiopica*.

Table 2. Fatty acid composition of individual lipid classes of thylakoid membranes from leaves and regreened spathes of *Z. aethiopica*

Lipid	Mol% of total fatty acid						
	16:0	16:1-c	16:1-*tr*	18:0	18:1	18:2	18:3
MGDG							
Leaf	3	1	—	1	2	2	90
Spathe	5	2		2	2	4	85
DGDG							
Leaf	15	1	—	2	2	6	73
Spathe	15	1		2	2	8	72
SQDG							
Leaf	38	1	—	2	7	17	35
Spathe	35	1		2	7	15	40
PG							
Leaf	29	1	30	1	1	3	34
Spathe	41	3		4	8	9	35
PC							
Leaf	24	4		3	10	13	44
Spathe	23	4		4	9	14	43
Total							
Leaf	12	1	2	2	2	5	75
Spathe	15	2	—	2	4	7	70

Fig. 1. Ultrastructural aspects of chloroplast from leaf of *Z. aethiopica* showing typical arrangement of thylakoid membranes in grana (arrows) and non-grana regions. S—starch. Bar 1 μm.

Fig. 2. Detail of palisade cell from regreened spathe of *Z. aethiopica* showing well-structured chloroplasts with well-organized grana regions (arrows). S—starch. Bar—1 μm.

Several authors have reported that some preparations of LHCII made by SDS solubilization, followed by SDS-PAGE, are enriched in PG and 16:1-*tr* [16, 17]. These authors suggested the existence of specific interactions between PG and LHCII, that could be responsible for the formation of the oligomeric form of this pigment-protein complex. It was also mentioned that the enzymatic removal of PG from purified LHCII resulted in a complete conversion of the oligomeric to the monomeric form on SDS-PAGE separation [32]. The monomeric form of LHCII has also been reported to re-associate into the oligomeric form in 16:1-*tr* containing PG liposomes [17, 33].

Other workers have reported a mutant of *Arabidopsis thaliana* lacking 16:1-*tr* that did not exhibit apparent changes in chloroplast structure or changes in the properties of LHCII, although comparison of the electrophoretic patterns of Chl-protein complexes from the wild-type and the *fadA* mutant (lacking C16:1-*tr*) of *A. thaliana* revealed that the mutant lacked LHCP[1] [21, 22]. According to these authors, 16:1-*tr* is not important in conferring unique functional properties to LHCII and they suggested that the reported changes in the ratio of LHCII[1]:LHCII[3] could be artifacts of the detergent

solubilization of thylakoid membranes. According to the same authors, 16:1-*tr* would have a more subtle role in optimizing the efficiency of photosynthetic electron transport, this molecule being an element of the fine-tuning mechanisms for control of energy transfer. In addition, some plant species with no 16:1-*tr* in PG still have normal ratios of LHCII oligomer to monomer [19].

After detergent solubilization, thylakoid membranes from leaf and regreened spathes of *Z. aethiopica* showed identical electrophoretic patterns of Chl-protein complexes. The four bands observed were identified as CP1, LHCP[1], CPa and LHCP[3] (data not shown). The presence of the LHCP[1] band in regreened spathe thylakoids, which is believed to represent the oligomeric form of LHCP, suggests that in *Z. aethiopica* the presence of 16:1-*tr* is not important for the establishment of the oligomeric form of LHCII. These results agree with the fact that there is no obvious differences in the size and extent of grana in regreened spathes when compared with leaf chloroplasts.

With regard to photochemical activities, it was found that regreened spathe thylakoids exhibited lower values of PSII and PSI activities (64.3 and 119.6 μmol O_2 hr^{-1} mg^{-1} Chl, respectively) compared to the values of PSII and PSI activities (99 and 354 μmol O_2 hr^{-1} mg^{-1} Chl, respectively) in leaf thylakoids. According to our results, a possible role for 16:1-*tr* is in LHCII stabilization, resulting in an increase of energy transfer efficiency from LHCII to the PSII reaction centre. The lower values of photochemical activities in regreened spathe thylakoids could also be explained by the lower degree of unsaturation of these membranes, which could correspond to a lower degree of membrane fluidity. Nevertheless, some authors have stated that there is no consistent correlation between unsaturation and diffusion rates [34].

In spite of the absence of 16:1-*tr*, the regreened spathe chloroplasts are functional and exhibit structural organization similar to the leaf chloroplasts. This suggests that there is no correlation between granal stacking and the presence of 16:1-*tr* in *Z. aethiopica*.

EXPERIMENTAL

Plant material. Leaves and regreened spathes were collected from *Z. aethiopica* Spreng. plants growing in the Lisbon Botanical Garden.

Isolation of thylakoid membranes. All steps were carried out at 4° using a modified procedure described earlier [35]. Freshly harvested and de-veined leaves, and regreened spathes, were homogenized in a Waring blender with 10 mM tricine–NaOH pH 6.5 containing 330 mM sorbitol, 5 mM $MgCl_2$, 1 mM benzamidine, 0.1% (v/v) 2-mercaptoethanol and 1% (w/v) PVP (ratio of plant material to homogenization buffer was 1:4 (w/v)). The crude homogenate was filtered through 8 layers of cheesecloth and one layer of nylon cloth (20 μm pore size) and centrifuged at 1000 *g* for 2 min. The pellet was re-suspended and washed ×2 in homogenization buffer for 10 min at 1000 *g*. The washed pellet was re-suspended in 1 mM tricine–NaOH pH 6.5 containing 5 mM $MgCl_2$. After incubation for 5 min, the suspension was centrifuged for 10 min at 1000 *g*. The pellet was finally re-suspended in a small vol. of the same osmotic shock medium and chlorophyll determined according to ref. [36]. The purity of isolated thylakoid membranes was determined by transmission electron microscopy, according to the procedure described below.

Lipid and fatty acid analysis. Lipids were extracted from freshly prepd thylakoids, with $CHCl_3$–MeOH (1:2) [37] and stored in $CHCl_3$ at −10° ([Chl] = 5 mg ml^{-1}). Lipid classes were separated by 2D TLC on precoated silica gel plates, using 100 μl of lipid extract. Plates were developed in Me_2CO–benzene–MeOH–H_2O (8:3:2:1) in the first dimension and in $CHCl_3$–MeOH–aq. NH_3 (13:7:1) in the second dimension [38]. After spraying the plates with 0.2% (w/v) 8-aniline-4-naphthosulphonic acid in MeOH, lipids spots were detected under UV light (365 nm). Identification of lipid spots was made by spraying the plates with various chromogenic reagents [39] and by comparison of R_f values with co-chromatographed lipid standards (Sigma). To quantify lipids and to determine their fatty acid composition, the silica gel corresponding to each spot was scraped off and subjected to transesterification in MeOH–benzene–H_2SO_4 (20:1:1). The corresponding fatty acid Me esters (FAME) were analysed by GC and GC-MS.

GC. The chromatograph equipped with a FID detector, a data-handling system and a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temp. was prog. from 150° to 190° at 3° min^{-1}, held isothermal for 12 min at 190° and then prog. to 280° at 5° min^{-1}. Inj. and det. temps were 300°; carrier gas H_2 was adjusted to a linear velocity of 300 cm sec^{-1}. Samples were injected using the split-sampling technique with a ratio of 1:150. The concentration of each FAME was computed from GC peak areas using correction factors. Response factors were calculated relative to Me 20:0.

GC-MS. The chromatograph was equipped with a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm) and interfaced with an ion-trap detector. Oven conditions were as described above; transfer line temp. 280°; ion-trap temp. 220°; carrier gas He adjusted to a linear velocity of 30 cm sec^{-1}; split ratio 1:40; ionization energy 70 eV; ionization current 60 μA; scan range 40–500 mu; scan time 1 sec. Identify of FAME was assigned by comparison of their R_t values and MS with the corresponding data of ref. FAME [40]. Determination of double bond position in monounsaturated FAME was carried out on the corresponding dimethyl disulphide adducts [41]. The data shown represent the average of five independent expts performed on five different samples of leaf and regreened spathe thylakoids. Standard errors were less than 7%.

Electron microscopy. Leaf and regreened spathe palisade parenchyma was cut into small pieces (1 mm^3) and fixed with 2% (w/v) formaldehyde, 2.5% (v/v) glutaraldehyde in 50 mM Na cacodylate buffer pH 7.2, for 3 hr at 4°. After three washes (10 min each) in the same fixative

buffer, samples were post-fixed with 1% (w/v) OsO_4 in 50 mM Na cacodylate buffer pH 6.8, for 2 hr at room temp. The material was then dehydrated in an Me_2CO series and embedded in Epon–Araldite [42]. Ultrathin sections were stained with uranyl acetate–lead citrate [43] and observed in an electron microscope at 80 kV.

REFERENCES

1. Allen, C. F. and Good, P. (1971) *Meth. Enzymol.* **23**, 523.
2. Chapman, D. J. and Barber, J. (1987) *Meth. Enzymol.* **148**, 294.
3. Siegenthaler, P., Rawley, A. and Smutny, J. (1989) *Biochim. Biophys. Acta* **975**, 104.
4. Dorne, A.-J., Joyard, J. and Douce, R. (1990) *Proc. Natl Acad. Sci. USA* **87**, 71.
5. Gounaris, K., Barber, J. and Harwood, J. L. (1986) *Biochem. J.* **237**, 313.
6. Harwood, J. L. (1980) in *The Biochemistry of Plants Lipids: Structure and Function* (Stumpf, P. K., ed.), pp. 1–55. Academic Press, New York.
7. Haverkate, F., De Gier, J. and Van Deenen, L. L. M. (1964) *Experientia* **20**, 511.
8. Dubacq, J. P. and Trémolières, A. (1983) *Physiol. Vég.* **21**, 293.
9. Harwood, J. L. (1975) in *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T. and Mercer, E. I., eds), pp. 43–93. Academic Press, New York.
10. Galey, J., Francke, B. and Bahl, J. (1980) *Planta* **149**, 433.
11. Mackender, R. O. (1979) *Plant Sci. Lett.* **16**, 101.
12. Guillot-Salomon, T., Douce, R. and Signol, M. (1973) *Plant Sci. Lett.* **1**, 43.
13. Guet, C., Trémolières, A. and Lecharny, A. (1982) *Photochem. Photobiol.* **35**, 283.
14. Lechowicz, W., Maternicka, K., Faltynowickz, M. and Poskuta, J. (1986) *Photosynthesis Res.* **8**, 133.
15. Trémolières, A., Roche, O., Dubertret, G., Guyon, D. and Garnier, J. (1991) *Biochim. Biophys. Acta* **1051**, 286.
16. Trémolières, A. Dubacq, J. P., Ambard-Bretteville, F. and Remy, R. (1981) *FEBS Lett.* **130**, 27.
17. Remy, R., Trémolières, A., Duval, J. C., Ambard-Bretteville, F. and Dubacq, J. P. (1982) *FEBS Lett.* **137**, 271.
18. Huner, N. P. A., Krol, M., Williams, J. P., Maissan, E., Low, P. S., Roberts, D. and Thompson, J. E. (1987) *Plant Physiol.* **84**, 12.
19. Huner, N. P. A., Williams, J. P., Maissan, E. E., Myscich, E. G., Krol, A., Laroche, E. and Singh, J. (1989) *Plant Physiol.* **89**, 144.
20. Bolton, P., Wharfe, J. and Harwood, J. L. (1978) *Biochem. J.* **174**, 67.
21. McCourt, P., Browse, J., Watson, J., Arntzen, C. J. and Sommerville, C. R. (1985) *Plant Physiol.* **78**, 853.
22. Browse, J., McCourt, P. and Sommerville, C. R. (1985) *Science* **227**, 763.
23. Pais, M. S. (1972) *Port. Acta Biol. (A)* **12**, 101.
24. Chaves das Neves, H. and Pais, M. S. S. (1980a) *Tetrahedron Lett.* **21**, 4387.
25. Chaves das Neves, H. and Pais, M. S. S. (1980b) *Biochim. Biophys. Res. Commun.* **95**, 1387.
26. Pais, M. S. and Chaves des Neves, H. (1983) *Plant Growth Reg.* **1**, 233.
27. Pais, M. S., Novais, M. C. and Abreu, I. (1976) *Port. Acta Biol. (A)* **15**, 1.
28. Pais, M. S. S. (1981) *Ann. Sci. Nat., Bot., Paris* **13**, 109.
29. James, A. T. and Nichols, B. W. (1966) *Nature* **210**, 372.
30. Tuquet, C., Guillot-Salomon, T., De Lubac, M. and Signol, M. (1977) *Plant Sci. Lett.* **8**, 59.
31. Lemoine, Y., Dubacq, J.-P. and Zabulon, G. (1982) *Physiol. Veg.* **20**, 487.
32. Krupa, Z., Huner, P. N. A., Williams, J. P., Maissan, E. and James, D. R. (1987) *Plant Physiol.* **84**, 19.
33. Remy, R., Trémolières, A. and Ambard-Bretteville, F. (1984) *Photobiochem. Photobiophys.* **7**, 267.
34. Van Ginkel, G., Van Langen, H. and Levine, Y. K. (1989) *Biochimie* **71**, 23.
35. Anderson, J. M. and Andersson, B. (1985) *Cell Components* (Linskens, H. F. and Jackson, J. K., eds), pp. 231–258. Springer, Berlin.
36. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.
37. Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **31**, 911.
38. Sato, N., Nemato, Y. and Furuya, M. (1988) *Plant Physiol. Biochem.* **26**, 93.
39. Kates, M. (1972) *Techniques of Lipidology.* North-Holland, Amsterdam.
40. McCloskey, J. A. (1969) *Meth. Enzymol.* **14**, 382.
41. Buser, H.-R., Arn, H., Guerin, P. and Rauscher, S. (1983) *Anal. Chem.* **55**, 818.
42. Mollenhauer, H. H. (1964) *Biochim. Biophys. Acta* **39**, 111.
43. Venable, J. H. and Coggeshall, R. (1965) *J. Cell Biol.* **25**, 407.