Molecular Species of Phosphatidylglycerol from Leaves of Various *Citrus* Species

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Phosphatidylglycerol (PG) is a phospholipid characteristic of chloroplasts of higher plants. Molecular species of PG isolated from leaves of 7 *Citrus* species were examined and grouped into 5 classes. More than 90% of the total molecules were of the prokaryotic type synthesized in chloroplasts. Fatty acid compositions (*sn-1/sn-2*) of the major molecular species were 18:1/16:1 (26-47% of the total molecules), 18:1/16:0 (11-35%), and 16:0/16:1 (10-22%), which were all prokaryotic. Molecular species with 18:1 at *sn-1* were characteristic of *Citrus* leaf PG. Unsaturated prokaryotic molecular species of (18:1, 18:2, and 18:3)/(16:0 and 16:1) accounted for 65-82%, and saturated ones of (16:0 and 18:0)/(16:0 and 16:1) for 19-32%.

Phospholipids are one of the major lipid classes found in membranes of higher plant leaves. Recent development of analytical methods for lipids enabled us to identify molecular species of phospholipids readily,¹⁻³⁾ which suggests that molecular species composition is more important than fatty acid composition of individual lipid classes. It is rather difficult to estimate molecular species compositions from fatty acid compositions.^{4,5)} Some particular molecular species of PG are important in establishing the thermal phase transition temperature in herbaceous plant leaves.^{6,7)} Therefore, molecular species analyses of lipids are required for further understanding of relationships between chemical structure and function of membrane systems.

The genus *Citrus* contains some of the most important fruit trees cultivated throughout the world. Most *Citrus* species are evergreen woody plants and further improvements in photosynthetic activity and chilling resistance leading to higher yields and better quality of the fruits

are desired. PG is a phospholipid characteristic of chloroplasts in leaves⁸⁾ and attention has been paid to this phospholipid in connection with photosynthetic activity⁹⁾ and chilling sensitivity.⁶⁾ However, little is known about molecular species of phospholipids in evergreen woody plant leaves such as *Citrus* species in spite of its importance.¹⁰⁻¹²⁾ This paper describes the molecular species composition of PG isolated from leaves of six *Citrus* species.

Materials and Methods

Plant materials. Leaves of *Citrus junos* cv. Tanenashimukaku yuzu were harvested at the Agricultural Experimental Station, Okayama University, in August; leaves of *C. grandis* (Tosa-buntan) in a greenhouse of our university in March; *C. unshiu* (unshu orange) on a local commercial farm in August; leaves of *C. sinensis* (Trovita orange) in the Akitsu Branch of the Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Japan in August; leaves of *C. paradisi* (grapefruit) and *C. sinensis* (navel orange) in a greenhouse of the ARCO Plant Cell Research Institute in Dublin, California, in November; *C. paradisi* (Pomelo) in the Botanical Garden of University of the Philippines at Los Baños, Philippines, in

Abbreviations: CL, cardiolipin; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglyceride; 16:0, palmitic acid; 16:1, (*E*)-3-hexadecenoic acid; 17:0, margaric acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

January; leaves of *C. amblycarpa* (Djeroek limoe) on a commercial citrus farm in Bandung, Indonesia, in August.

Chemicals. *Naja naja* venom phospholipase A₂, *Bacillus cereus* phospholipase C, phospholipids (PC, PE, PI, PS, CL, and PA) and PC (18:2/18:2, 16:0/18:2, 18:1/18:1, 16:0/18:1, 18:0/18:1, 18:1/18:0, 18:1/16:0, 18:0/18:0, and 16:0/16:0 for *sn*-1/*sn*-2) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.), and PC (18:3/18:3, 18:0/16:0, and 16:0/18:0) from Avanti Polar-Lipids, Inc. (Pelham, AL, U.S.A.). PC (18:0/16:1) was synthesized chemically from lyso-PC (*sn*-1, 18:0) and 16:1. 3,5-Dinitrobenzoylchloride was purchased from Wako Chemical Industries, Ltd., Tokyo. Precoated silica gel TLC plates were products of E. Merk (Silica gel 60) and Whatman Inc. (PLK5), and DEAE-Toyopearl 650M (Fractogel DEAE-650M) from Tosoh Ltd., Tokyo. All organic solvents used were glass-distilled.

Extraction and separation of lipids. Lipids were extracted from *Citrus* leaves essentially by the method of Allen and Good.¹³⁾ Total lipids obtained were separated into individual lipid classes by two-dimensional TLC on a Silica gel 60 plate and the fatty acid composition of each lipid was identified. PG was purified by preparative TLC on a PLK5 plate⁵⁾ or by column chromatography with DEAE-Toyopearl 650M coupled with the elution system described previously.¹⁴⁾ Each lipid class was confirmed by comparison of the *R_f* value with those of authentic specimens and spinach leaf lipids, and coloring reagents such as Dittmer's reagent for phosphorus groups, anthrone reagent for sugar residues, Dragendorff reagent for choline residues, and ninhydrin reagent for amino groups.^{5,15)}

Fatty acid analysis. Fatty acid constituents of each lipid sample were transesterified with 5% H₂SO₄ in MeOH for 2 hr at 70°C and then analyzed with GLC by the method described previously.¹⁵⁾

Analysis of positional distribution of fatty acids. After treatment of PG with phospholipase A₂, lysophosphatidylglycerol and free fatty acids were separated by TLC and then the fatty acid composition of each position was analyzed by the method described previously.¹⁵⁾

Preparation of 3,5-dinitrobenzoyl derivatives of PG. Purified PG or authentic PC specimens were first hydrolyzed by phospholipase C and the resulting 1,2-diacylglycerides were esterified with 3,5-dinitrobenzoylchloride in anhydrous pyridine as described previously.^{1,15)}

HPLC analysis of 3,5-dinitrobenzoyl derivatives. 3,5-Dinitrobenzoyl derivatives of PG were dissolved in small amounts of the mobile phase and injected into an HPLC mounting Nucleosil 3C18 column (4.6 × 150 mm). Elution was achieved isocratically with acetonitrile–2-propanol (80:20, v/v). Each peak was collected and the fatty acid composition was analyzed by the method described above. Retention times of the peaks were compared with those of 3,5-dinitrobenzoyl derivatives of authentic specimens, phospholipids from pollen grains of trumpet lily (*Lilium longiflorum*)⁵⁾ and tea,¹⁵⁾ and PG from tea leaves.⁴⁾

Results and Discussion

Lipid composition

Citrus leaves contained 20–30 mg of extractable lipids per 1 g fresh weight. Major glycerolipids found in *Citrus* leaves were MGDG, DGDG, SQDG, PI, PG, PC, and PE, when a two-dimensionally developed TLC plate was stained with I₂ vapor. PS, PA, and CL were also detected as minor ones. A typical glycerolipid composition of *C. junos* was shown in Table I. Seven glycerolipids listed in

Table I. CONTENTS AND FATTY ACID COMPOSITIONS OF MAJOR Glycerolipids ISOLATED FROM *C. junos* LEAF

Lipid class	Content ^a (μmol/g fresh wt)	Fatty acid composition (mol%)					
		16:0	16:1	18:0	18:1	18:2	18:3 Others
MGDG	1.73 ^b (37) ^c	8	0	3	3	5	80 1
DGDG	1.10 (23)	36	0	16	4	3	39 2
SQDG	0.61 (13)	49	0	15	10	4	20 2
PG	0.37 (8)	29	27	10	29	2	2 1
PC	0.26 (6)	47	1	14	20	8	8 2
PE	0.17 (4)	37	0	13	16	17	13 4
PI	0.44 (9)	56	0	6	9	10	16 3

^a Total lipids extracted were 21.3 mg/g fresh weight.

^b Calculated from fatty acid composition by using 17:0 as an internal standard.

^c Values in parentheses indicate % distribution within the lipids listed.

Table II. FATTY ACID COMPOSITIONS OF PG ISOLATED FROM LEAVES OF VARIOUS *Citrus* SPECIES

Species	Fatty acid composition (mol%)						Others
	16:0	16:1	18:0	18:1	18:2	18:3	
Arachicitrus TANAKA							
<i>C. grandis</i> (Tosa-buntan)	26	36	2	18	10	8	tr. ^a
<i>C. paradisi</i> (Grapefruit)	34	20	4	30	7	3	2
<i>C. paradisi</i> (Pomelo)	27	26	6	35	3	tr.	3
<i>C. sinensis</i> (Navel orange)	27	30	4	30	3	1	5
<i>C. sinensis</i> (Trovia orange)	29	31	4	34	1	1	0
Metacitrus TANAKA							
<i>C. junos</i> (Yuzu)	32	32	8	24	tr.	2	2
<i>C. unshiu</i> (Unshu)	26	29	4	33	4	4	0
<i>C. amblycarpa</i> (Djerook limoe)	39	29	5	19	5	3	0

^a Detectable but less than 0.5%.

Table I accounted for about 20% of total lipids extracted. Fatty acid compositions of the seven glycerolipids were analyzed after the individual lipid classes were separated by two-dimensional TLC. Major fatty acid constituents were 18:3 for MGDG; 16:0, 18:0, and 18:3 for DGDG and SQDG; 16:0, 16:1, and 18:1 for PG; 16:0, 18:0, and 18:1 for PC; 16:0, 18:0, 18:1, 18:2, and 18:3 for PE; and 16:0, 18:1, 18:2, and 18:3 for PI. Significant amounts of fatty acids with carbon numbers of 14, 20 and more were not found in these glycerolipids.

Leaf PG in various *Citrus* species

Fatty acid compositions of leaf PG were further examined in various *Citrus* species. Leaf PG occurred mostly in the chloroplast as well as MGDG, DGDG, and SQDG.⁸⁾ PG was isolated from leaves by DEAE-Toyopearl 650M column chromatography followed by silica gel column chromatography. PG was one of the major phospholipids in all *Citrus* species examined. The fatty acid compositions of leaf PG obtained were similar among the various species examined (Table II). A large amount of 18:1 was present in PG isolated from *Citrus* leaves. This agreed with earlier work done with *Citrus* species,¹⁰⁾ tea,⁴⁾ and some woody plants,¹⁶⁾ although 18:1 accounted for less than 10% of the total fatty acid constituents

Table III. POSITIONAL DISTRIBUTION OF FATTY ACIDS IN PG ISOLATED FROM *C. junos* LEAF

Position	Fatty acid composition (mol%)					
	16:0	16:1	18:0	18:1	18:2	18:3
<i>sn</i> -1	29	2	16	50	1	1
<i>sn</i> -2	20	63	5	5	2	2

in most herbaceous plants.^{12,14,17,18)} Three fatty acids (16:0, 16:1, and 18:1) accounted for 80–90% of the total constituents in 8 kinds of *Citrus* leaves (Table II). The fatty acid constituents 16:0, 16:1,^{*1} and 18:0 were 58–64% of the total fatty acids and polyunsaturated 18:2 and 18:3 were minor ones, probably due to use of leaves grown under warm condition.

16:1 was exclusively located in the *sn*-2 position of the glycerol moiety and 18:1 in the *sn*-1 position (Table III). These results agreed with earlier work.^{4,12,19)} There was no apparent difference in fatty acid compositions between the two classes of *Citrus* species, arachicitrus and metacitrus (Table II).

Molecular species of PG

Isolated PG was converted to 3,5-dinitrobenzoyl derivatives and analyzed by HPLC. A typical elution profile of the derivatives

*1 Fatty acid (16:1) with an (*E*)-3-double bond was classified in the same group as 16:0 because 16:1 is closely related to 16:1 with respect to biosynthesis¹⁹⁾ and thermal phase transition²⁰⁾ in PG.

prepared from *C. junos* leaf PG is shown in Fig. 1. Each peak was collected and the fatty acid composition was analyzed by GLC after transmethylation. Retention times of the peaks were also compared with those of 3,5-dinitrobenzoyl derivatives with known fatty acid compositions. We also assumed that the

Fig. 1. HPLC Profile of 3,5-Dinitrobenzoyl Derivatives of PG Isolated from Leaves of *C. junos* Grown in Summer. Peak numbers are corresponding to Tables IV and V.

majority of 18:1 was located at *sn*-1 of the glycerol moiety and 16:1 at *sn*-2. The evidence for identification of each peak is summarized in Table IV.

Molecular species compositions of PG are shown in Table V. PG molecules isolated from leaves of the various *Citrus* species were grouped in 5 classes biosynthetically based on fatty acid composition (*sn*-1/*sn*-2); 18:m/18:n (<4% of the total molecules), 18:m/(16:0 and 16:1) (65–82%), 18:0/(16:0 and 16:1) (3–7%), 16:0/(16:0 and 16:1) (14–28%), and 16:0/18:m (<9%), where the numbers of m and n are 1, 2, and 3. Among them, 18:m/(16:0 and 16:1), 18:0/(16:0 and 16:1), and 16:0/(16:0 and 16:1), which are so-called prokaryotic molecules,²¹⁾ are synthesized in the chloroplast. These 3 classes of PG molecules accounted for more than 90% of the total molecules in all species examined (Table V). Thus, PG isolated from the leaves reflects

Table IV. IDENTIFICATION OF HPLC PEAKS OF 3,5-DINITROBENZOYL DERIVATIVES OF PG

Peak ^a no	Relative retention time	Evidence ^b	Fatty acid composition (mol%)						Fatty acid <i>sn</i> -1/ <i>sn</i> -2
			16:0	16:1	18:0	18:1	18:2	18:3	
1	0.33	A, B	n.d. ^c						18:3/18:3
2	0.40	B	n.d.						(18:2/18:3) ^d
3	0.45	D	8	56	0	0	0	36	18:3/16:1
4	0.49	A, B	n.d.						18:2/18:2
5	0.52	B, D	32	9	4	4	0	56	18:3/16:0 ^e
6	0.54	D	5	36	0	7	50	2	18:2/16:1
7	0.57	n.d.	n.d.						
8 ^f	0.69	A, B, D	31	5	15	0	36	13	18:2/16:0 ^g
		D							(18:3/18:0)
9	0.77	C, D	5	41	1	47	5	1	18:1/16:1
10	0.82	D	51	35	3	6	4	1	16:0/16:1
11	0.94	A, D	47	1	2	47	2	1	18:1/16:0
12	1.00	A, D	85	0	0	8	7	0	16:0/16:0
13	1.05	A, D	8	42	38	0	12	0	18:0/16:1
14	1.38	A	n.d.						(18:0/16:0) ^h

^a Peak numbers are the same as those in Fig. 1.

^b A, comparison of the retention time with those of authentic specimens; B, comparison of the retention time with those of pollen lipids of lily⁵⁾ and/or tea¹⁵⁾; C, comparison of the retention time with those of the tea leaf PG derivatives⁴⁾; D, fatty acid analysis.

^c Not determined.

^d Mixture of 18:2/18:3 and 18:3/18:2.

^e A possibility of the presence of 16:0/18:3 could not be excluded.

^f Mixture of 18:2/16:0, 18:3/18:0 and 18:0/18:3.

^g A possibility of the presence of 16:0/18:2 could not be excluded.

^h Mixture of 18:0/16:0 and 16:0/18:0.

Table V. MOLECULAR SPECIES COMPOSITIONS OF PG FROM VARIOUS *Citrus* LEAVES

Peak no	Fatty acid		Molecular species composition (mol%)						
	C-1	C-2	<i>C. grandis</i>	<i>C. paradisi</i> (Pomelo)	<i>C. sinensis</i> (Navel)	<i>C. sinensis</i> (Trovita)	<i>C. junos</i>	<i>C. unshiu</i>	<i>C. amblycarpa</i>
1	18:3	18:3	tr. ^a	2	1	1	2	tr.	2
2	(18:2/18:3) ^b		2	0	2	tr.	0	tr.	0
3	18:3	16:1	10	4	3	4	6	3	7
4	18:2	18:2	1	0	1	0	tr.	tr.	0
5	18:3	16:0	3	2	2	1	1	2	3
6	18:2	16:1	9	4	5	5	5	2	5
7	nd		tr.	0	0	tr.	tr.	tr.	tr.
8	18:2	16:0 ^b	6	2	3	1	1	3	1
	(18:3/18:0) ^b								
9	18:1	16:1	26	32	36	47	43	29	46
10	16:0	16:1	22	11	11	11	14	10	10
11	18:1	16:0	11	34	23	21	17	35	20
12	16:0	16:0	6	7	9	4	4	9	4
13	18:0	16:1	3	2	3	4	6	4	4
14	18:0	16:0	1	1	1	1	1	3	1

^a Detectable but less than 0.5%.^b See footnotes in Table IV.

chloroplast PG.

Major molecular species of PG common in all species examined were 18:1/16:1 (26–47%), 18:1/16:0 (11–35%), and 16:0/16:1 (10–22%). In addition *C. grandis* contained 18:3/16:1 (10%) and 18:2/16:1 (9%), and *C. sinensis* (navel) and *C. unshiu* 16:0/16:0 (9%). These major molecular species were all prokaryotic. Occurrence of 18:1/(16:0 and 16:1) in large amounts (37–68%) is characteristic of *Citrus* leaf PG as well as leaf PG of evergreen woody plants, tea (40–70%),⁴⁾ and avocado (46%).¹²⁾ The sum of prokaryotic unsaturated molecular species, 18:1/(16:0 and 16:1) and (18:2 and 18:3)/(16:0 and 16:1), accounted for 65–82% of the total molecules, while saturated species, 16:0/(16:0 and 16:1) and 18:0/(16:0 and 16:1) were 19–32% (Table V). Thus, the ratios of unsaturated/saturated molecular species of *Citrus* leaf PG presented here are similar to those of chilling-resistant herbaceous plants such as pea and lettuce rather than chilling-sensitive herbaceous plants.⁶⁾

In Table II, relative contents of saturated fatty acid constituents range from 58 to 64%

(Table II), although those of saturated molecular species were 19–32%. This indicates that estimation of molecular species composition from whole fatty acid composition is very difficult. Therefore, fatty acid compositions of glycerolipids in some earlier works should be re-examined in terms of molecular species compositions. Further investigation of the relationship between response to environmental stress and molecular species composition is now in progress.

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