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Transmembrane topography of plasma membrane constituents in mung bean (*Vigna radiata* L.) hypocotyl cells I. Transmembrane distribution of phospholipids

Yuichi Takeda, Kunihiro Kasamo *

Research Institute for Bioresources, Okayama University, 1-20-2 Chuo, Kurashiki 710-0046, Japan

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Abstract

The transmembrane distribution of phospholipids (PLs) in the plasma membrane (PM) of mung bean (*Vigna radiata* L.) hypocotyl cells was investigated using annexin V-fluorescein isothiocyanate, porcine pancreas phospholipase A_2, and ^{31}P-nuclear magnetic resonance (NMR) spectroscopy. Phosphatidylserine was not located on the cell surface of mung bean protoplasts. However, phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid were found to be almost symmetrically distributed across right-side-out PM vesicles obtained by aqueous two-phase partitioning by porcine pancreas phospholipase A_2 assay. ^{31}P-NMR assay showed that the amount of PLs is about equal in the outer and the inner leaflets of the right-side-out PM vesicles. These results suggest that the topography of PM PLs might not contribute to well-known asymmetrical properties of the outer and inner surfaces of higher plant PMs. It is also indicated that inside-out PM vesicles created by Brij 58-treatment do not retain the native PL topography on dithionate reduction of 7-nitro-2,1,3-benzoxadiazol-4-yl-labeled PLs incorporated in the PM vesicles.

Keywords: Plasma membrane; Higher plant cell; Asymmetry; Phospholipid; Protoplast; Two-phase partitioning

1. Introduction

The plasma membrane (PM) in higher plants is the outer border of the cells and plays important roles in various cellular processes such as the uptake of nutrients and ions, redox processes, growth regulation, hormone activities, endo- and exocytosis, host–pathogen interactions, and many kinds of stress responses [1–5]. In the last two decades, many studies have been carried out to reveal the functions and properties of plant PMs [1] accompanying the development of isolation procedures [6,7].

Several articles have reported that the properties of the outer surface of higher plant PMs were distinctly different from those of the inner surface. For instance, right-side-out and inside-out PM vesicles from higher plant tissues can be separated by an aqueous two-phase partition technique [7–10] and free-flow electrophoresis [11]. Endomembrane vesicles from rye seedlings aggregated on addition

Abbreviations: FITC, fluorescein isothiocyanate; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PM, plasma membrane

* Corresponding author. Fax: +81-86-434-1249;
E-mail: kasamo@rib.okayama-u.ac.jp

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of $ZnCl_2$ or lowering of the pH to below 5, while right-side-out PM vesicles did not [12]. The outer surface of the PM vesicles from various species and the tissues of higher plants have a lower isoelectric point and a lower charge density than the inner surface [13,14]. Such differences between the surfaces of exofacial (apoplastic site) and cytofacial leaflets in plant PMs should be derived from the asymmetric distribution of the membrane constituents, namely, phospholipid(PL)s, sterols, glycolipids, oligosaccharides and/or surface peptides including peripheral proteins. It is suggested that among these components the oligosaccharides of glycoproteins and the glycolipids might be exposed on the outer surface of plant PM [1,11]. However, the topography of other membrane constituents and the cause of the asymmetrical properties in the plant PM remain obscure. Undoubtedly, some of the PM functions are closely related to its asymmetry [15].

In this study, we investigated the transmembrane distribution of PLs and indicated its poor contribution to the asymmetrical surface properties in higher plant PM.

2. Materials and methods

2.1. Chemicals

Dextran T500 and T70 were purchased from Amersham Pharmacia. Polyethylene glycol 3350, phospholipase A_2 (porcine pancreas), and annexin V-FITC conjugate from Sigma, and (1-oleoyl-2-NBD-aminododecanoyl) ((oleoyl-C_{12}-NBD))-PLs from Molecular Probes. All other reagents were of analytical grade.

2.2. Plant material

Seeds of the mung bean (*Vigna radiata* L.) were hydrated in tap water and grown in the dark at 33°C. The hypocotyls were excised from 2-day-old etiolated seedlings and chilled in ice-cold water until use.

2.3. Protoplast isolation and annexin V-FITC staining

Protoplasts were prepared from mung bean hypocotyls as described elsewhere [16] and finally suspended in 10 mM MES–Tris (pH 7.3) containing 0.5 M mannitol and 1 mM $CaCl_2$.

Annexin V-FITC and propidium iodide were added to the protoplast suspension to final concentrations of 4 μg ml^{-1} and 1 μg ml^{-1}, respectively, and allowed to stand for 5 min in the dark at room temperature. Annexin V peculiarly binds to PS in the presence of Ca^{2+} ions and its dye-conjugate is universally used for detection of apoptotic cells [17,18]. Propidium iodide was used for dead-cell staining. Protoplasts heated at 70°C for 7 min were used as a positive control.

Phase-contrast and dye fluorescence microscopic images were taken using an Olympus (Tokyo, Japan) IX70 microscope equipped with a cooled CCD camera.

2.4. Membrane preparations

PM vesicles were obtained from mung bean hypocotyls by two-phase partitioning using dextran T500 and polyethylene glycol 3350 [19,20]. Excised hypocotyl sections (150 g fresh weight) were homogenized in 300 ml of grinding medium containing 0.25 M sucrose, 50 mM MOPS–KOH (pH 7.6), 5 mM EGTA, 1.5% (w/v) PVP, 0.2% (w/v) BSA, 0.2% (w/v) casein (boiled for 10 min), 2 mM PMSF, and 2.5 mM $K_2S_2O_5$. The 12 500–100 000 × *g* pellet was suspended in 0.25 M sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8), then added to a phase mixture with a final composition of 6.2% (w/w) dextran T500, 6.2% (w/w) polyethylene glycol 3350, 0.25 M sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8) (36 g final weight). The U_3+$U_{3'}$ (upper phase) highly enriched in right-side-out PM vesicles and the L_1 (lower phase) enriched in endomembrane vesicles after a batch procedure [7] were diluted with suspension buffer containing 0.25 M sucrose, 10 mM MES–Tris (pH 7.3), and 1 mM DTT and centrifuged at 100 000 × *g* for 30 min. The resulting pellets were washed and pelleted again to remove the remaining polymers. The final pellets were resuspended with suspension buffer. All procedures for membrane preparation were conducted on ice or at 4°C. Protein content was determined by the method of Bradford [21] using the Bio-Rad assay reagent with BSA as the standard. The membrane preparation and protein assay were completed within 6 h.

The high purity of PM vesicles from the U_3+$U_{3'}$ was verified by assays for ATPase activity with various inhibitors [22], latent IDPase and UDPase, NADH-cytochrome *c* reductase, and cytochrome *c* oxidase [23] (data not shown). Freshly prepared PM vesicles were generally 85–90% latent for PM ATPase activity, assessed with 0.015% (w/v) Triton X-100.

Unless otherwise indicated, unfrozen, freshly prepared membrane vesicles were immediately used in assays for PL distribution.

2.5. Analytical methods for lipid quantification

Lipids were extracted by the method of Bligh and Dyer [24]. The suspension of membranes was boiled for 3 min prior to the extraction. The lipid extracts were separated by two-dimensional thin-layer chromatography (TLC) using development solvents as shown by Yoshida and Uemura [25]. The spots on the TLC plate were visualized by spraying 0.01% (w/v) primuline in acetone/water (4:1, v/v) and illuminating with UV light. The area corresponding to each lipid was scraped, transferred into a test tube and quantified.

PLs were quantified by the method of Rouser et al. [26]. Cerebroside and galactolipids, and sterols were respectively quantified according to Roughan and Batt [27], and Zlatkis and Zak [28] using glucose and galactose, and cholesterol as the standards.

2.6. Treatment of right-side-out PM vesicles with porcine pancreas phospholipase A_2

Right-side-out PM vesicles at 1 mg protein ml^{-1} in suspension buffer containing 10% (w/v) dextran T70 were incubated at 30°C under gentle agitation with 5 U ml^{-1} porcine pancreas phospholipase A_2 and 1 mM $CaCl_2$ for 0 to 30 min. At various incubation times, aliquots were taken and mixed with the same volume of suspension buffer containing 50 mM EDTA to stop the reaction, then immediately boiled for 3 min to inactivate both exogenous and endogenous phospholipases completely. After cooling, lipids were extracted and separated by two-dimensional TLC with tetrahydrofuran/methanol/acetic acid/water (50:20:40:8, by volume) for the first dimension and chloroform/acetone/methanol/acetic acid/water (100:40:20:30:10, by volume) for the second. The remaining PC, PE and PA were quantified. Parallel reactions were run without the phospholipase A_2 because plant membranes generally have activities of Ca^{2+}-dependent phospholipases, especially high phospholipase D activity [25,29], which modified the PL composition of PM during the incubation mixtures (see Section 3). Therefore, correction for the contribution of endogenous phospholipases was done using the following formula, assuming that the PLs were modified by both endogenous phospholipases and the exogenous phospholipase A_2:

$$\%\text{modified lipid by porcine pancreas phospholipase } A_2 = \%\text{ total modified lipid} - \%\text{ modified lipid by endogenous phospholipases} \quad (1)$$

0.03% (w/v) Triton X-100 was used to determine the total pool of PLs in both leaflets accessible to the phospholipase A_2.

2.7. ^{31}P-NMR measurement

^{31}P-Nuclear magnetic resonance (NMR) spectra were obtained with a Varian VXR-500S spectrometer operating at 202.3 MHz in a pulsed Fourier transform mode at 16°C without spinning. The pulse angle was 45° and the pulse repetition time was 0.20 s. The number of scan times was 12 032–16 384.

Right-side-out PM vesicles, both fresh and stored at −80°C, in suspension buffer containing 15% (v/v) ^2H_2O were incubated in a 10-mm diameter NMR tube. The protein content was 7–10 mg ml^{-1}. After the spectrum had been recorded, $PrCl_3$, a paramagnetic ion, was added from a concentrated stock solution into the incubation mixture (final 10 mM) to separate the ^{31}P signal due to PL in the outer leaflet from that in the inner leaflet [30,31]. Its spectrum was then recorded. The change in volume of the incubation mixture was less than 10%. Methylene diphosphonic acid (80 mM) in a small glass capillary vessel placed in the tube was used for comparison of the peak areas. 0.1% (w/v) Triton X-100 was utilized to render all phosphorus in the PLs accessible to the paramagnetic ions.

2.8. The preparation of PM vesicles containing (oleoyl-C_{12}-NBD)-PLs and dithionate assay

Concentrated stock solutions of (oleoyl-C_{12}-NBD)-PC, -PE and -PS were prepared in absolute ethanol. To incorporate NBD-PLs into the outer leaflet, an aliquot of the ethanolic solution was taken and added to 1 ml of the suspension of right-side-out PM vesicles (1 mg of protein) with a final concentration of 1–2 nmol NBD-PLs/mg protein and stood for 15 min at room temperature in the dark. The suspension was diluted with suspension buffer and centrifuged at 100 000×*g* for 30 min at 4°C. The pellets were washed and centrifuged again to completely remove unincorporated NBD-PLs. The resulting PM pellet containing NBD-PLs was resuspended in 1 ml of suspension buffer.

To invert into inside-out PM vesicles, 1 ml of the suspension of the right-side-out PM vesicles (1mg of protein) containing NBD-PLs was mixed with 1 ml of suspension buffer containing 1% (w/v) Brij 58 [32] and stood for 15 min at room temperature. The suspension was diluted 15-fold with suspension buffer. The labeled inside-out PM vesicles were recovered by centrifugation at 100 000×*g* for 30 min at 4°C repeated twice and resuspended in 1 ml of suspension buffer. This procedure for the preparation of inside-out PM vesicles using Brij 58 was defined as 'Brij 58-treatment'.

The transmembrane location of NBD-PLs in right-side-out or inside-out PM vesicles was determined from the reduction of NBD moiety by dithionate [33]. PM vesicles containing NBD-PLs (0.1–0.2 mg of protein) were incubated in 2 ml of 135 mM KCl, 10 mM MES–Tris (pH 7.2) in a fluorescence cuvette. The fluorescence intensity was monitored with a spectrofluorophotometer (model RF-5300PC, Shimadzu, Kyoto, Japan) at 37°C with excitation and emission wavelengths of 490 and 540 nm, respectively. Ten μl of 1 M sodium dithionate (in 1 M Tris) was added to the incubation mixture and the fluorescence intensity was recorded. The decrease in fluorescence intensity will be induced as the result of the reduction of NBD-PLs in the outer leaflet [33]. Then, Triton X-100 was added with a final concentration of 0.2% (w/v) to disrupt the vesicles and quench the fluorescence due to NBD-PLs in the inner leaflet.

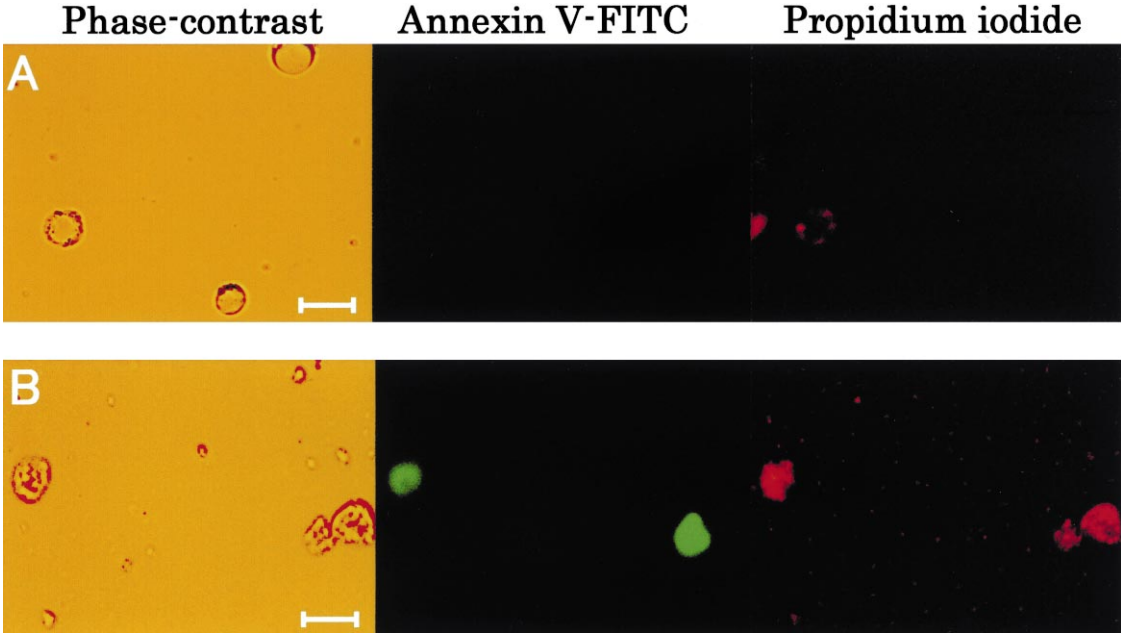

Fig. 1. Annexin V-FITC staining of protoplasts from mung bean hypocotyls. Non-heated (upper panels, A) and heated protoplasts (lower panels, B) were stained with annexin V-FITC and propidium iodide. Phase-contrast and dye fluorescent microscopic images of the same specimens are shown. Bars = 50 μm.

3. Results

3.1. Topography of PS

It has been reported that apoptosis occurred in plant cells as well as animal cells and that PS appeared on the cell surface at the early stage of apoptosis in tobacco cells [18], indicating that PS is also located in the inner leaflet of the plant PM in the same way as in mammalian cells [34]. We confirmed that PS was preferentially distributed in the inner leaflet of mung bean PM as shown in Fig. 1.

Most of the non-heated protoplasts were vital (Fig. 1A, right) and little fluorescence originating from FITC was detected (Fig. 1A, middle), suggesting that PS does not exist on the cell surface. On the other hand, all the heated protoplasts were dead (Fig. 1B, right) and green fluorescence due to FITC was detected in most of the heated protoplasts (Fig. 1B, middle). It is considered that annexin V-FITC became permeable across the membranes of the heated cells and was bound to PS in the inner leaflet of the PM and/or the endomembranes.

3.2. Lipid analysis of mung bean PM

The lipid composition of mung bean PM vesicles before treatment with porcine pancreas phospholipase A_2 is summarized in Table 1. The obtained data on a mol% basis were in good agreement with those reported by Yoshida and Uemura [25]. PLs and sterols were the major components of mung bean PM lipids, and PC, PE and PA were the major PLs composing 89.5% of total PL. Lyso-PC was not detected in the PM preparation. Monogalactosyldiglyceride and digalactosyldiglyceride, the major lipids of the plastid membrane, were also undetectable, suggesting few cross-contaminants in the membrane.

Table 1
Lipid composition of plasma membrane vesicles isolated from mung bean hypocotyls

Lipids	μmol mg^{-1} protein	mol%
Total PLs	0.68 ± 0.03	51.9
PC	0.23 ± 0.01	17.4
PE	0.28 ± 0.01	21.2
PA	0.10 ± 0.01	7.9
PS	0.04 ± 0.00	2.7
PI	0.02 ± 0.00	1.9
PG	0.01 ± 0.00	0.8
Lyso-PC	Not detected	–
Total sterols[a]	0.59 ± 0.01	44.8
Cerebroside	0.04 ± 0.00	3.2
Monogalactosyldiglyceride	Not detected	–
Digalactosyldiglyceride	Not detected	–
Total	1.28 ± 0.05	100.0

Means (±S.E.) of three different preparations are given on both a μmol mg^{-1} protein basis and a mol% basis.
[a]Includes free sterols, acylated sterylglycoside and sterylglycoside.

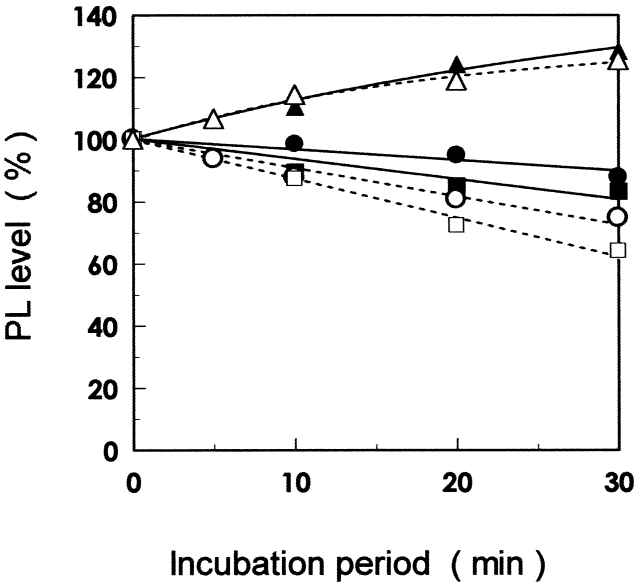

Fig. 2. Self-digestion of mung bean PM PLs by endogenous phospholipases. Freshly prepared right-side-out PM vesicles (filled symbols and solid lines) or those frozen at −20°C for over 1 month (open symbols and dashed lines) were incubated in the presence of 10% (w/v) dextran T70 and 1 mM $CaCl_2$ at 30°C. Triton X-100 was added to the suspension of frozen PM to a final concentration of 0.03% (w/v). Changes in PC (●, ○), PE (■, □), and PA (▲, △) contents at various incubation times were expressed as a percentage of those at time zero. Typical profiles of the self-digestion are presented.

3.3. Transmembrane distribution of the three major PLs in right-side-out PM vesicles

In order to ascertain whether a higher plant PM has a specific topography of PLs, the transmembrane distributions of PC, PE and PA were investigated with porcine pancreas phospholipase A_2. Other minor PLs could not be assayed because of their scarcity.

Fig. 2 shows that, in the absence of exogenous

phospholipase A_2, PC and PE slightly decreased while PA increased in the presence of 1 mM $CaCl_2$ at 30°C even in the buffer of pH 7.3, due to endogenous phospholipase D. Therefore, correction was done by Eq. 1 (see Section 2) to calculate the net PLs hydrolyzed by porcine pancreas phospholipase A_2.

Because plant membranes contain a PL pool inaccessible to the probes for PL asymmetry [35,36] correlated with protein to lipid ratios [35], it is necessary to evaluate the pool accessible to phospholipase A_2 in mung bean PM for the determination of PL topography. For this purpose, 0.03% (w/v) Triton X-100, sufficient to make membrane vesicles permeable but insufficient to solubilize membrane proteins, was used in order to render the PLs in both leaflets accessible to the phospholipase A_2. However, the presence of such a surfactant in the incubation mixture of freshly prepared PM resulted in marked self-digestion of the PLs by endogenous phospholipases (data not shown). Therefore, PM vesicles were frozen at −20°C for over a month to reduce endogenous phospholipase activities even in the presence of Triton X-100 (Fig. 2) and were used for the determination of the accessible pool.

The time courses of PL hydrolysis in right-side-out PM vesicles from mung bean hypocotyls by porcine pancreas phospholipase A_2 are shown in Fig. 3. The measurements made with Triton X-100 and frozen PM provided an estimation of the total accessible pool in both leaflets. And 58.8% of PC, 57.0% of PE, and 94.2% of PA, calculated from the exponentially fitted curves, were found to be accessible to the phospholipase A_2. In the experiments with sealed right-side-out PM vesicles, each PL was gradually degraded by phospholipase A_2 and the decrease reached stable levels except that the degradation profile of PE was biphasic. It is suggested that the hydrolysis of PC and PA in the outer leaflet reached a plateau within 30 min, while the first phase of the PE degradation corresponded to the hydrolysis of PE in the outer leaflet and the second phase to further hy-

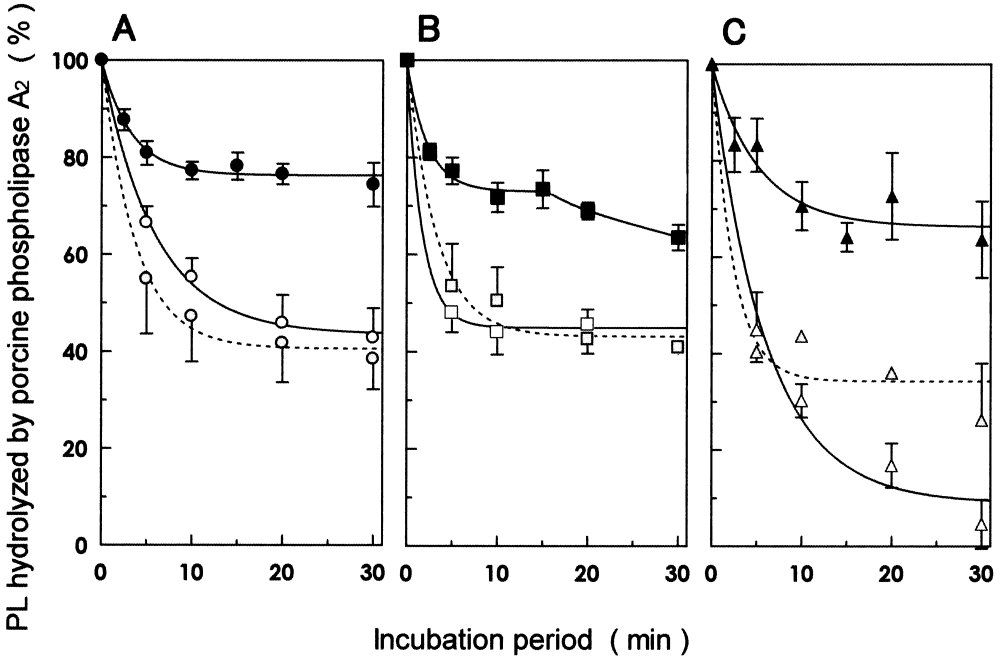

Fig. 3. Hydrolysis of PLs in mung bean PM by porcine pancreas phospholipase A_2. Freshly prepared right-side-out PM vesicles (filled and gray symbols) or those frozen at −20°C for over 1 month (open symbols) were incubated with phospholipase A_2 and 1 mM $CaCl_2$ in the presence (filled and open symbols, solid lines) or absence (gray symbols, dashed lines) of 10% (w/v) dextran T70. Triton X-100 (0.03% (w/v)) was added to the suspension of frozen PM before the reaction was started. PL contents hydrolyzed by phospholipase A_2 at various incubation times were expressed as a percentage of those at time zero with a correction by Eq. 1, as described in Section 2. (A) PC; (B) PE; (C) PA. Error bars indicate S.E. of 3–5 different preparations. Curves were fitted to the exponential function except for the second phase of PE hydrolysis of fresh PM vesicles (B, time 15–30 min).

drolysis of PE due to a transbilayer movement from the inner leaflet and/or a transfer from the inaccessible pool in the outer leaflet. The accessible pools of PC, PE and PA in the outer leaflet of right-side-out PM vesicles were estimated to be 23.9%, 27.2%, and 33.9%, respectively.

In this experiment, dextran T70 was necessary to maintain the integrity of the PM vesicles during the enzymatic treatment. Hovius et al. [37] also used dextran T40 for the phospholipase A_2-treatment of rat liver mitochondria to prevent the loss of membrane integrity. Omission of dextran T70 in the incubation of PM vesicles resulted in loss of the integrity due to the phospholipase A_2-treatment, and the time course of PL hydrolysis by phospholipase A_2 was similar to that with Triton X-100 and frozen PM except that PA was less degraded (Fig. 3). It seemed that Triton X-100 made PA more accessible to the phospholipase A_2. The hydrolyzed PLs in the absence of dextran T70 were PC, 59.4%; PE, 65.8%; PA, 65.7%, which would also reflect the total accessible pool.

Taken together, PC, PE, and PA seem to be almost symmetrically distributed across the mung bean PM, although the inaccessible pools cannot be attributed to the outer or inner leaflet.

The PL distribution assay of mung bean PM had been difficult. We also tried phospholipase A_2 from bee venom and phospholipase C from *Bacillus cereus*, but use of these phospholipases resulted in rapid collapse of the vesicles even in the presence of dextran T70. Plant PM seemed to be more fragile on exposure to these enzymatic treatments than mammalian membranes. The chemical probes for labeling of aminophospholipids, 2,4,6-trinitrobenzenesulfonate and fluorescamine [38], were found to be ineffective (data not shown). PE in both leaflets of the PM in appropriate buffers was poorly labeled by 2,4,6-trinitrobenzenesulfonate, and fluorescamine easily penetrated the mung bean PM.

Another approach to the analysis of PL topography was taken using ^{31}P-NMR spectroscopy. The transmembrane distribution of PL can be assayed utilizing paramagnetic ions such as Mn^{2+}, Eu^{3+}, and Pr^{3+} [30]. In sealed right-side-out PM vesicles in the incubation mixture at 16°C, Pr^{3+} was considered membrane-impermeable. Ten mM $PrCl_3$ did not aggregate right-side-out PM vesicles, although endomembrane vesicles (L_1) were aggregated by 1 mM $PrCl_3$.

Fig. 4A shows the ^{31}P-NMR spectrum of right-side-out PM vesicles from mung bean hypocotyls. A large, broad asymmetric peak (white arrow) and narrow symmetric peaks were observed. The former originated from PM PLs, the latter from free Pi and other unknown phosphorus compound contaminants (Fig. 4A, X) in the suspensions.

In the presence of both $PrCl_3$ and 0.1% (w/v) Triton X-100, only a broad and small peak was observed (Fig. 4C). Under this condition, all the phosphorus should be affected by the paramagnetic ions. The high-field peak from PM PL at −11 ppm (Fig.

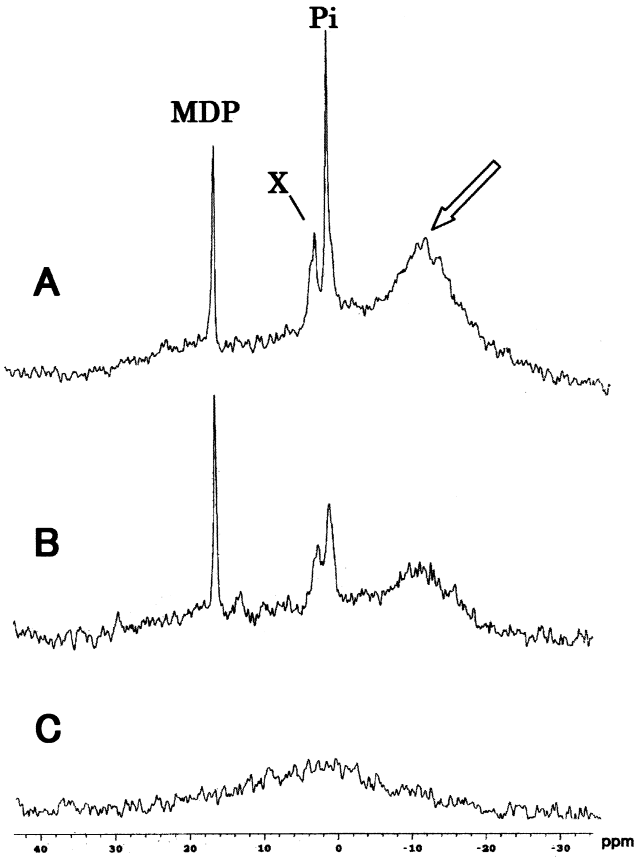

Fig. 4. ^{31}P-NMR spectra of right-side-out PM vesicles from mung bean. The spectra were obtained at 16°C with a Varian VXR-500S spectrometer. The peak position of 85% orthophosphoric acid was set at 0 ppm. Spectra shown are of right-side-out PM vesicles with no additives (A), after addition of 10 mM $PrCl_3$ (B), and in the presence of both 10 mM $PrCl_3$ and 0.1% (w/v) Triton X-100 (C). MDP, methylene diphosphonic acid; X, unidentified phosphorus compound(s). In spectrum C, the MDP capillary vessel was not utilized.

4A, white arrow) shifted to a higher magnetic field by 12 ppm. However, under our experimental conditions, a large part of the signal was diminished by binding of the broadening agents to the PLs (Fig. 4C). The signals from the free phosphorus compounds were also lost in the baseline or the small peak.

Therefore, the residual peak of the high-field peak from −24 ppm to −5 ppm was compared before and after the addition of 10 mM $PrCl_3$ (Fig. 4A,B), assuming that in sealed vesicles the PL molecules in the inner leaflet were not accessible to the metal.

Fig. 4B shows that addition of 10 mM $PrCl_3$ reduced the high-field peak of PL to nearly half (52.7%), suggesting that the quantity of total PLs in the outer leaflet is nearly equal to that in the inner leaflet.

Although the topography of each PL species could not be determined by this NMR experiment, it does not seem that the pool of PM PLs inaccessible to porcine pancreas phospholipase A_2 (Fig. 3) mainly originated from the PLs in only one leaflet.

3.4. Evaluation of membrane vesicles for the PL distribution assays

The occurrence of PL randomization during membrane preparation seems unlikely in tightly sealed PM vesicles. As shown in Fig. 5A, more than 90% of fluorescent PL analog, (oleoyl-C_{12}-NBD)-PLs, was incorporated in the outer leaflet of right-side-out PM vesicles by the procedure described under Materials and methods. This distribution of NBD-PLs did not change up to 8 h incubation at 4°C, and only 3–5% of NBD-PLs in the inner leaflet had increased after 23–25-h incubation (result not shown). It is suggested that PLs in tightly sealed membrane vesicles undergo little flip-flop movement, at least, at low temperature.

The PL distribution assay with inside-out PM vesicles should be efficient to truly evaluate the result of the PL degradation of right-side-out PM vesicles by porcine pancreas phospholipase A_2 presented in Fig. 3. Inside-out PM vesicles may be obtained both by freeze/thawing and repeating the two-phase step and by Brij-treatment [10,32], however, inside-out PM vesicles of mung bean suitable for the assay of PL distribution could not have been obtained.

First, inside-out PM vesicles were poorly formed from right-side-out PM vesicles by freeze/thawing even in the presence of high concentration of KCl [10] or even if osmotic gradients were made across the membrane vesicles [32]. These procedures for the preparation of inside-out PM vesicles cannot be nec-

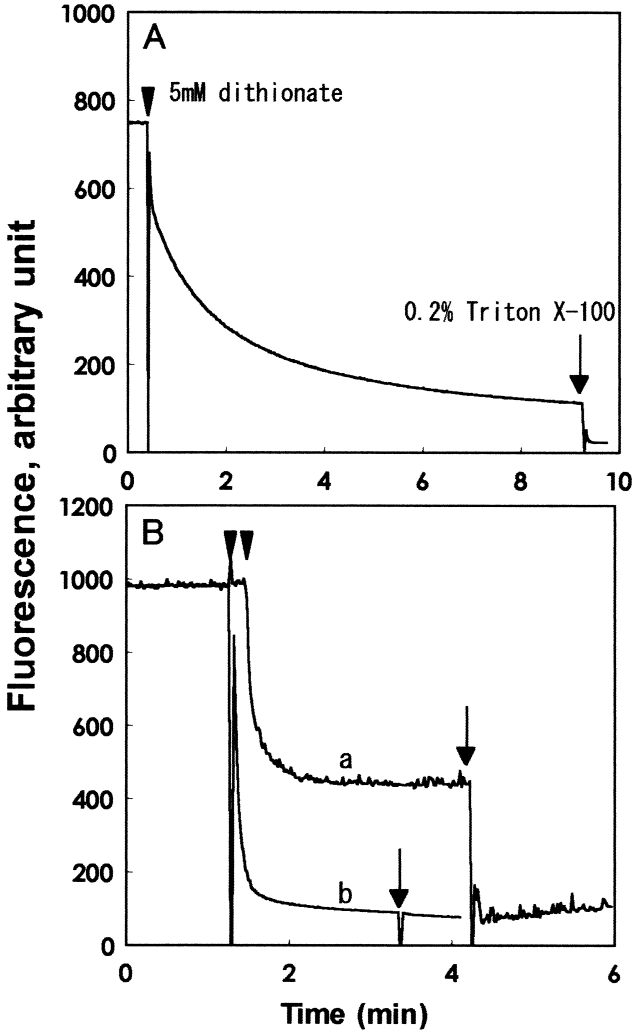

Fig. 5. Reaction of dithionate with PM vesicles containing (oleoyl-C_{12}-NBD)-PS. NBD-PS was incorporated into the outer leaflet of right-side-out PM vesicles, and then inside-out PM vesicles were created by Brij 58. The detergent was later removed by centrifugation. Fluorescence intensity of the right-side-out PM vesicles (A) and the inside-out PM vesicles (B) containing NBD-PS was recorded. In both A and B, 5 mM sodium dithionate was added at the time indicated by the arrowheads. After several minutes, 0.2% (w/v) Triton X-100 was added at the time indicated by the arrows. Traces in B: trace b, in the presence of 0.5% (w/v) Brij 58 in the fluorescence cuvette; trace a, in the absence of Brij 58. The same results were obtained using (oleoyl-C_{12}-NBD)-PC and -PE instead of -PS.

essarily applied to all the plant PMs. Moreover, it is known that a large number of freeze/thawing cycles leads to loss of tightness of membrane vesicles. Leaky vesicles would not be suitable for the phospholipase A_2-treatment because they would easily loose integrity on enzymatic treatment.

Second, it was indicated that the Brij 58-treatment might randomize the PL distribution of right-side-out PM vesicles. Fig. 5B (trace a) shows that NBD-PLs are present in nearly equivalent amounts in the outer and inner leaflet of inside-out PM vesicles created by the Brij 58-treatment from the right-side-out PM vesicles, having been predominantly located in the outer leaflet before the treatment (Fig. 5A). Furthermore, in the presence of 0.5% (w/v) Brij 58 in the fluorescence cuvette, all the NBD-PLs in the inside-out PM vesicles were reduced by dithionate as if Brij 58 disrupted the vesicles in the same way as Triton X-100 (Fig. 5B, trace b). When the right-side-out PM vesicles containing NBD-PLs predominantly in the outer leaflet (Fig. 5A) were incubated with 0.5% Brij 58 in the cuvette, the profile of fluorescent quenching by dithionate was similar to that in Fig. 5B, trace b.

4. Discussion

In this report, we assayed the transmembrane distribution of PLs across mung bean PM to examine whether the cause of the asymmetrical surface properties in higher plant PMs is derived from the specific topography of PM PLs.

The general trend for mammalian cells is that choline-containing PLs, PC and sphingomyelin, are preferentially located in the outer leaflet of PM, and amino-PLs, PE and PS, in the inner leaflet [34,39]. PA and PI also reside in the inner leaflet in the erythrocyte membrane [34]. However, we demonstrated that the transmembrane distribution of PLs in the plant PM was different from that of mammalian PMs. PC, PE and PA would be almost symmetrically distributed across right-side-out PM vesicles from mung bean hypocotyls (Fig. 3), although some PA might be generated from other PLs, mainly PC and PE, during the membrane isolation [25,29].

Umeda and Emoto [40] reported that the transbilayer movement and redistribution of PM PE was necessary for regulation of actin filament assembly during cell division, presumably via membrane protein, in Chinese hamster ovary cells. Therefore, it is plausible to consider that remarkable PL asymmetry, at least with PE, might not be required for mature plant hypocotyl cells where cell division does not occur. Otherwise, plant PM might not utilize the transbilayer movement of PLs for the control of cytoskeleton. It is considered that plant PM, at least of the tissues in mature regions, does not need to generate and maintain transmembrane asymmetry of the major PLs for physiological purposes and the PLs seem to be symmetrically distributed by passive diffusion.

PS was located in the inner leaflet of plant PM the same as in mammalian PMs (Fig. 1). This asymmetry of PM PS seems to be a common feature in all normal cells of living creatures [34,41–43]. The PS asymmetry and its regulation is considered important to cellular processes such as cell-cell recognition [15,34], endo- and exocytosis [39], regulation of membrane surface potential [43], and activation of membrane-bound enzymes [15,44], as indicated in mammalian and yeast cells. The PL asymmetry of mammalian PM may be controlled by PL flippase, namely ATP-dependent amino-PL translocase [34,39,45,46], whereas the amino-PL translocase is not necessarily related to the generation and maintenance of the PL asymmetry [46–48]. The physiological significance of the PS asymmetry in plant PMs is still unclear, and the existence of PL flippase in the plant membrane has not yet been shown. We tried to detect PL flippase (floppase) activity in the inside-out PM vesicles created by Brij 58-treatment using the (oleoyl-C_{12}-NBD)-PLs (Fig. 5B, trace a) under various conditions, varying the buffer components and compositions, temperature, the NBD-PLs/protein ratio, and the reaction periods, but we could not find any ATP-dependent PL translocating activity, although PS was located in the inner leaflet (Fig. 1). It is probable that a mechanism to retain PS in the inner leaflet of PM, not based on the amino-PL translocase, exists in plant as well as mammalian cells. For instance, it is possible that there might be a specific interaction between PS and the membrane cytoskeleton [15] or a membrane protein in the monolayer [49].

Brij 58 is a useful tool to obtain inside-out PM vesicles [32], however, in the present study it was

indicated that the Brij 58-treatment might randomize the PL distribution (Fig. 5B, trace a) in spite that it may arrange the direction of PM proteins. It is enigmatic that in the presence of 0.5% Brij 58, all fluorescence due to NBD-PLs was extinguished by dithionate (Fig. 5B, trace b) in spite that under the conditions sealed membrane vesicles were formed and ATP-dependent H^+-transport could clearly be seen [32]. Since the information presented in Fig. 5B is limited, further study is needed to identify what is truly happening to the PM, and PM lipids in particular, in the presence of Brij 58. One assumption is that inside-out PM vesicles scarce of PLs were formed and most of the PLs were solubilized by the high concentration of Brij 58.

In conclusion, it is considered that remarkable differences in surface properties between the two leaflets of the plant PM are not derived from the topography of the PLs because the major PLs including negatively charged PA are nearly symmetrically distributed across the PM (Fig. 3) whereas PS, the minor charged PL, was preferentially located in the inner leaflet (Fig. 1 and Table 1). The next paper in this series will show that other constituents of higher plant PM have outstanding transmembrane asymmetry that would cause the difference in surface properties.

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