FEBS Letters 498 (2001) 32–36 FEBS 24900

# Phosphatidylserine delivery to endoplasmic reticulum-derived vesicles of plant cells depends on two biosynthetic pathways

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Received 12 March 2001; revised 2 May 2001; accepted 3 May 2001

First published online 15 May 2001

Edited by Ulf-Ingo Flügge

**Abstract Vesicles formed from endoplasmic reticulum (ER) by a cell-free system of leek cells (*Allium porrum*) are enriched in phosphatidylserine (PS), especially species containing very long chain fatty acids (VLCFA, at least 20 carbon atoms). In plant cells, PS is formed either by PS synthase or the serine exchange enzyme, although it is not known which pathway(s) contribute(s) to PS delivery in the ER-derived vesicles (EV), nor to what extent this occurs. Taking advantage of a cell-free system, we have shown that PS enrichment originates mainly from the serine exchange enzyme which is the only pathway that synthesizes the VLCFA-PS species. On the other hand, both enzymes synthesize PS with long chain fatty acids (up to 18 carbon atoms), but these species are given to the EV by PS synthase.** 

*Key words:* Plant cell; Endoplasmic reticulum-derived vesicle; Phosphatidylserine synthesis and delivery

## 1. Introduction

A central problem in cell biology consists in understanding how the endomembrane system of eukaryotic cells is involved in the synthesis, sorting, and transport of proteins and lipids for the biogenesis of plasma membranes.

A certain amount of progress has been made recently in plant cells concerning our knowledge of phosphatidylserine (PS) traffic to the cell surface where it is enriched, and between the endoplasmic reticulum (ER) and the Golgi [1]. A cell-free ATP-dependent transfer between these two organelles has been demonstrated in leek cells [2], mimicking an in vivo situation [3]. Recently, an ATP-dependent formation of PS-rich vesicles from the ER (EV) was also obtained in vitro [4]. These vesicles were characterized by a mean average size of 70 nm, as already observed in many eukaryotic organisms [5–9], and accumulated very long chain fatty acid (VLCFA) PS species [4]. These results suggested that specific delivery of PS took place at the ER budding level.

Contrary to animal and yeast cells which have only one PS biosynthetic pathway [10–12], plant cells are distinguished by the presence of CMP-phosphatidic acid (CMP-PA): L-serine 3-phosphatidyltransferase (PS synthase: CMP-PA+serine → PS+CMP), and phosphatidyl-X:L-serine 3-phosphatidyltransferase (serine exchange enzyme: phosphatidylcholine (PC)/phosphatidylethanolamine (PE)+serine → PS+choline/ethanolamine), [13,14]. With the cell-free system [4], we have determined experimental conditions allowing the synthesis of PS by only one biosynthetic pathway at a time, and investigated their contribution to the PS enrichment in the EV. What happened to PS was compared with what happened to phosphatidylinositol (PI), which was taken as a reference because similar levels of it are to be found in the ER membranes [1] and because it does not accumulate in the EV [4]. We have also determined which PS species are synthesized by each pathway.

Taking into account the acyl chain content of PS in the EV [4], our results show that PS enrichment noticed in the vesicle originates mainly from the serine exchange enzyme which synthesizes and delivers VLCFA-PS, and that PS synthase synthesizes and delivers most of the LCFA-PS species.

## 2. Materials and methods

### 2.1. Plant material and chemicals

Leek (*Allium porrum* L.) seeds were purchased from Vilmorin (France). They were allowed to germinate, and seedlings were grown as described previously [4].

All the chemicals came from Sigma (St. Louis, MO, USA). L-[$^{14}$C(U)]Serine (166 Ci/mol), [2-$^{3}$H(N)]*myo*-inositol (22.1 Ci/mmol), [5-$^{3}$H]CTP (22,4 Ci/mmol), [α-$^{32}$P]CTP (3000 Ci/mmol) and [2-$^{14}$C]malonyl-CoA (58.3 Ci/mol) were supplied by DuPont NEN (Boston, MA, USA). L-3-PC 1-palmitoyl-2-[1-$^{14}$C]palmitoyl (55 Ci/mol), L-3-PE 1-palmitoyl-2-[1-$^{14}$C]palmitoyl (100 Ci/mol), L-3-PC and L-3-PE 1-palmitoyl-2-[1-$^{14}$C]linoleoyl (55 Ci/mol) came from Amersham, France.

### 2.2. Experimental conditions for PS and PI synthesis

ER membranes were prepared and characterized as described previously [4,15]. Protein concentrations were determined by the Bradford method [16], using bovine serum albumin (BSA) as the standard.

Experimental conditions for CTP:phosphatidate cytidylyltransfer-

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*Abbreviations:* ER, endoplasmic reticulum; EV, endoplasmic reticulum-derived vesicles; FAME, fatty acid methyl ester; HPTLC, high performance thin layer chromatography; LCFA, long chain fatty acids (up to 18 carbon atoms); PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; VLCFA, very long chain fatty acids (at least 20 carbon atoms)

0014-5793/01/$20.00 
PII: S0014-5793(01)02475-9

ase (CTP cytidylyltransferase), PS synthase, CMP-PA:*myo*-inositol 3-phosphatidyltransferase (PI synthase) and the serine exchange enzyme activities in the ER of leek seedlings were determined, using as a starting point those already performed on the ER of other plants [13].

The serine exchange enzyme was measured with 50 μg of ER membrane proteins in 0.1 M HEPES, pH 7.8, or 0.05 M Tris–HCl, pH 8.5, containing 10 mM β-mercaptoethanol, 3 mM $MgCl_2$ and/or 2 mM $MnCl_2$ and/or 10 mM $CaCl_2$ or without ions. The reaction was run with 1 μCi of L-[$^{14}$C(U)]serine for 30 min at 30°C in a final volume of 50 μl.

After neosynthesis of CMP-PA by CTP cytidylyltransferase as previously described [13], ER membranes were centrifuged at $150\,000 \times g_{max}$ for 15 min (Hitachi Himac CS 100), and used to measure all PS and PI synthesizing activities. In order to do this, membranes were treated as indicated for the serine exchange enzyme assay, or with 1.5 mM $MnCl_2$ and 0.5 μCi of [2-$^3$H(N)]*myo*-inositol. PS synthase activities were calculated by subtracting those of the serine exchange from both activities.

Optimal syntheses were obtained with protein amounts tested from 0.01 to 0.15 mg of ER membrane proteins, kinetics from 5 to 90 min, and substrate concentration from 5 μM to 2 mM serine or *myo*-inositol.

The reactions were stopped by adding three volumes of chloroform:methanol (2:1, v/v), and neosynthesized lipids were analyzed as indicated in Section 2.6.

### 2.3. PS delivery to ER-derived vesicles according to the two PS biosynthetic pathways

The cell-free system consisted of ER membranes (0.5 mg) containing PS synthesized by the serine exchange enzyme or PS synthase, and those (0.5 mg) containing PI synthesized by PI synthase. PS/PI ratios varying from 0.07 to 18 were tested for each PS biosynthetic pathway. ER membranes used for the synthesis of PS by the exchange enzyme were maintained at 30°C, and treated in a similar way to those used for the synthesis of CMP-PA required for PI synthesis.

Membranes were then pooled and centrifuged at $150\,000 \times g_{max}$ (Hitachi Himac CS 100) for 15 min at 4°C. Pellets were re-suspended in the buffer for the formation and isolation of the EV as reported earlier [4]. The radioactivity of both PS and PI was determined as indicated in Section 2.6, and an 'enrichment factor': PS/PI (EV)/PS/PI (ER) was calculated for each biosynthetic pathway by dividing the PS/PI ratio obtained for the vesicles by that measured for the ER membranes.

### 2.4. PS species synthesized by the serine exchange enzyme

To determine whether the serine exchange enzyme can synthesize VLCFA-PS, VLCFA were first made by the fatty acid elongase from [$^{14}$C]malonyl-CoA to produce labelled VLCFA-PC and -PE [17,18] (see Fig. 5). Total VLCFA content in lipids was analyzed with five aliquots (250 μg of ER membrane proteins). Lipids were separated [19], fatty acid methyl esters (FAMEs) were prepared [20] and analyzed as follows. The hexane phase was evaporated, FAMEs were re-suspended in 20 μl of chloroform:methanol (2:1, v/v) and analyzed on high performance thin layer chromatography (HPTLC) RP-18 $F_{254S}$ reverse-phase plates eluted with acetonitrile:tetrahydrofuran (80:20, v/v). Labelling of VLCFAs was determined with a phosphorimager (Molecular Dynamics SI, Pharmacia).

The ER membranes (50 μg of proteins) were then incubated with L-serine (2 mM) to synthesize PS as determined above. Putative acyl-remodelling reactions during this step were avoided or limited by washing ER membranes after VLCFA synthesis, in 0.1 M HEPES (pH 7.8) containing 10% BSA (w/v) followed by a centrifugation at $150\,000 \times g_{max}$ for 15 min (Hitachi Himac CS 100). Two controls, either without further incubation for PS synthesis or incubation without addition of serine, were carried out. The acyl chains of polar lipids were then separated and analyzed as indicated above.

For long chain fatty acid (LCFA) PS species, ER membranes (0.25 mg) re-suspended in 0.1 M HEPES, pH 7.8, were either sonicated or not for 1 min in the presence of 0.1 μCi of L-3-PC 1-palmitoyl-2-[1-$^{14}$C]palmitoyl, or 0.05 μCi of L-3-PE 1-palmitoyl-2-[1-$^{14}$C]palmitoyl, or 0.1 μCi of L-3-PC or L-3-PE 1-palmitoyl-2-[1-$^{14}$C]linoleoyl. After centrifugation at $150\,000 \times g_{max}$ for 15 min (Hitachi Himac CS 100), pellets were re-suspended for PS synthesis (with 50 μg proteins and 2 mM of L-serine). Polar lipids were then separated and analyzed as described in Section 2.6.

### 2.5. PS species synthesized by PS synthase

ER membranes (0.1 mg) were incubated with 0.5 mM of CTP containing 10 μCi of [α-$^{32}$P]CTP to synthesize labelled CMP-PA with CTP cytidylyltransferase. The reaction was stopped with two volumes of chloroform:methanol (1:1, v/v), and lipids were extracted and washed as described [19]. They were then separated onto HPTLC silica gel plates (Merck $60F_{254}$) by two-dimensional chromatography. The first elution was with chloroform:methanol:acetic acid:water (65:40:2:3, v/v), and the second with chloroform:methanol:water (60:35:8, v/v). Neosynthesized [α-$^{32}$P]CMP-PA was visualized with a Phosphorimager (Molecular Dynamics SI, Pharmacia), and scraped off the plate into screw-capped tubes for preparation of FAMEs [20]. An aliquot of the hexane phase (1 μl) was analyzed by gas liquid chromatography (GLC; Hewlett Packard 5890 Series II) on a 15 m × 0.53 mm Carbowax column (Altech). An initial temperature of 160°C was maintained for 1 min, and then increased up 180°C at 20°C/min. After 30 s, a secondary step was made up to 210°C at 3°C/min. After 30 s, a third step was made up to 245°C at 10°C/min and this final temperature was maintained for 10 min. FAME retention times were compared with those of standards.

### 2.6. Lipid analyses

Lipids were separated as described by Heape et al. [19]. After being stained with iodine vapor or revealed with a phosphorimager (Molecular Dynamics SI, Pharmacia), PS, PI, PC or PE were scraped off the plates into scintillation vials to determine their radioactivity by liquid scintillation counting in a Packard 2000 CA scintillation counter [4].

## 3. Results and discussion

### 3.1. Specific enzymatic assay conditions for PS and PI biosynthetic pathways

The ER of plant cells has two PS biosynthetic pathways: PS synthase and the serine exchange enzyme. The objective of this study was to investigate their contribution to the delivery of PS to EV. We first set specific assay conditions for each PS synthesizing activity. Since PS enrichment was measured in comparison with PI, assay conditions for PI synthesis were also determined.

HEPES buffer at pH 7.8 and $Ca^{2+}$ allowed a maximal serine exchange enzyme activity (14 nmol PS/mg protein/h) whereas it was considered preferable to use Tris–HCl at pH 8.5, $Mg^{2+}$ and $Mn^{2+}$ for PS synthase (4.5 nmol PS/mg protein/h). We have also determined that $Mg^{2+}$ is more efficient than $Mn^{2+}$, and sufficient in itself. Furthermore, each enzyme is not active when the assay conditions of the other one are used (data not shown), which only allows one activity to be measured at a time. PI synthase had optimal activity (about 9 nmol PI/mg protein/h) with Tris–HCl buffer at pH 8.5 with a preference for $Mn^{2+}$ in comparison with PS synthase.

CMP-PA made by CTP cytidylyltransferase with endogenous PA [1] guarantees that the lipid species neosynthesized are those normally formed by PS and PI synthases in vivo.

### 3.2. PS delivery to ER-derived vesicles according to the two PS biosynthetic pathways

To investigate the delivery of PS to EV, we used a cell-free system from leek cells which allows the formation and isolation of these vesicles [4]. By varying the L-serine and *myo*-inositol concentrations, we estimated PS enrichment in conditions where PS/PI ratios have the same values as those determined earlier in the ER: $0.51 \pm 0.12$ (lipid amounts) and $0.68 \pm 0.17$ (in vivo acetate labelling) [4]. An 'enrichment factor' of $2 \pm 0.3$ ($n = 5$) was observed when PS was synthesized by the serine exchange activity, and $4.3 \pm 0.1$ ($n = 4$) when PS was made by PS synthase (Fig. 1). These results indicate that

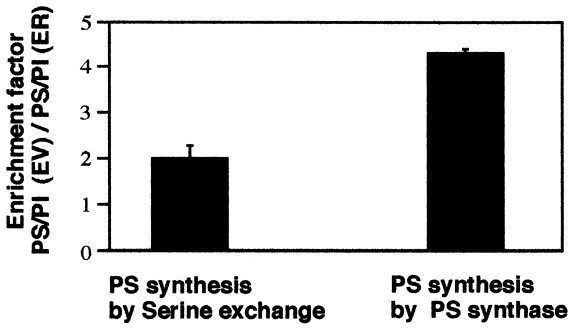


Fig. 1. Enrichment of PS to the ER-derived vesicles as a function of the two PS biosynthetic pathways in the ER of plant cells. For more details, see Section 2. Regardless of the PS/PI ratio in the ER, enrichment factors of $2 \pm 0.3$ ($n = 5$) were observed when PS was synthesized by the serine exchange activity, and $4.3 \pm 0.1$ ($n = 4$) when PS was made by PS synthase, and those are significantly different (with $P < 0.001$ according to Student's $t$-test).

PS formed by both biosynthetic pathways is delivered to the vesicles. We determined that PS cannot be synthesized by enzymes located in the vesicles [4]. Furthermore, the proportion delivered, as compared to PI, is higher after PS synthesis by PS synthase. To show that the comparison between the two PS synthesizing enzymes was accurate, we found that the 'enrichment factor' is independent of the level of synthesis of the phospholipids. Indeed, by varying the PS/PI ratio in the ER membranes for each biosynthetic pathway from 0.07 to 18 (mainly $< 1$ in order to show an effective PS enrichment), the values of the factors did not change.

### 3.3. PS species synthesized and delivered by the two biosynthetic pathways

We have previously analyzed the fatty acid content of PS in the EV (lipid amounts), and found three times as much VLCFA-PS species in comparison with the ER. This enrichment leads to a high content of VLCFAs which accounts for 34% of the total fatty acids [4].

PS species synthesized by PS synthase were investigated by GLC analyses of the fatty acyl chain content of neosynthesized CMP-PA, the direct precursor of PS synthesis by this pathway. The only fatty acids found were saturated $C_{16}$ and saturated/unsaturated $C_{18}$ (data not shown), indicating that no synthesis of VLCFA-containing CMP-PA had occurred. This result shows that PS synthase only synthesizes LCFA-containing PS.

To determine whether the serine exchange enzyme synthesizes LCFA-PS species, we sonicated ER membranes with labelled di-$C_{16:0}$ or $C_{16:0}$:$C_{18:2}$ species-containing PC and PE (Fig. 2). This treatment was necessary to observe a detectable level of PS synthesis. Under these conditions, we found that both phospholipids were used by the enzyme to produce PS (Fig. 2).

To determine whether the serine exchange enzyme synthesizes VLCFA-PS species from VLCFA-containing donors [17,18], we first synthesized VLCFAs and obtained 4 nmol of [$^{14}$C]VLCFAs/mg protein/h in ER membranes. Their subsequent incorporation into different lipids is shown in Fig. 3. Neutral lipids (diacylglycerols, triacylglycerols and free fatty acids) and PC exhibited a fatty acid label profile from saturated $C_{20}$ to $C_{28}$, but with a preferential accumulation of $C_{20}$. However, in PE, labelled fatty acids were saturated $C_{22}$ and

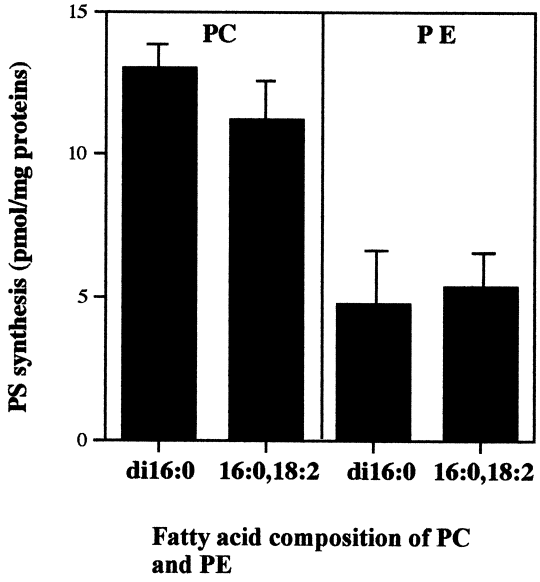


Fig. 2. LCFA-PS synthesis by the serine exchange enzyme from LCFA-PC and -PE species. The phospholipid substrates (275 000 dpm) were incorporated into ER membranes by sonication for 1 min. After centrifugation of the membranes, 5% of radioactivity was reproducibly incorporated for each substrate. After PS synthesis, lipids were separated on HPTLC plates [19] and analyzed by a phosphorimager. The data are means of three experiments ± S.D.

$C_{24}$ (Fig. 3). Therefore, we observed a selective incorporation of VLCFAs into lipids.

Incubation of ER membranes containing [$^{14}$C]VLCFA-PC and -PE with L-serine to synthesize [$^{14}$C]VLCFA-PS by the serine exchange did not alter the labelling of PC, but a significant decrease in the labelling of PE was correlated to a similar increase in labelled PS (Fig. 4). These results indicate that VLCFA-PE acts as the natural precursor for VLCFA-PS. Moreover, the analysis of the acyl chain content of

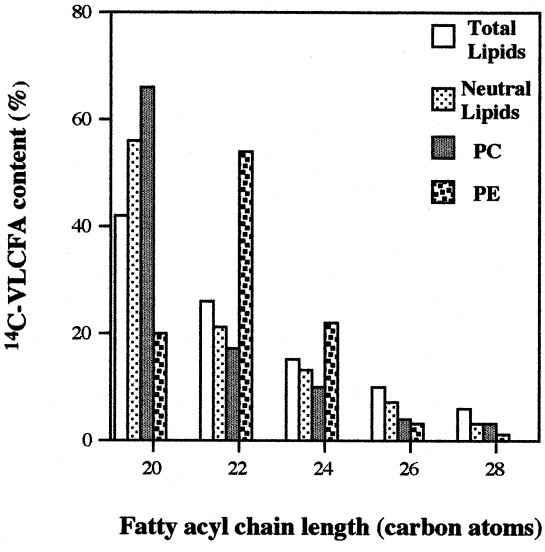


Fig. 3. VLCFA synthesis and incorporation into various lipid species. The percentage of total labelled VLCFA into lipids were obtained as described in Section 2. Data are means of two independent experiments with reproducible results, and show a selective incorporation of these fatty acid species.

[$^{14}$C]VLCFA-PS revealed the presence of saturated $C_{22}$ and $C_{24}$ exclusively (Fig. 4), which are the major acyl chains found in VLCFA-PS of the EV [4].

Our results give the conclusion that both biosynthetic pathways synthesize LCFA-PS but that only the serine exchange activity synthesizes VLCFA-PS.

Although there was sufficient labelling of VLCFA-PS in the ER to be analyzed (Fig. 4), we were not able to measure the amount of radioactivity associated with VLCFA-PS delivered to the EV (those vesicles representing only 5% of the total ER membranes). However, using the fatty acid content of PS in the vesicles [4], we could determine the proportion of PS species delivered by each biosynthetic pathway.

The facts that VLCFA phospholipid species mainly contain only one VLCFA esterified to the glycerol backbone and that PS in the vesicles contains about 30% VLCFA [4] lead to a VLCFA-PS/LCFA-PS ratio of 1.5 in the vesicles. On the other hand, the capability of the serine exchange enzyme to synthesize PS is three times that of PS synthase. Since the enrichment factor PS/PI (EV)/PS/PI (ER) of PS to the vesicles with the serine exchange enzyme is half that of PS synthase (Fig. 1), a ratio of 1.5 is also obtained between the amount of PS species from the serine exchange enzyme and PS synthase.

Moreover, the facts that VLCFA-PS species are only synthesized by the serine exchange enzyme and that PS synthase synthesizes exclusively LCFA-PS species lead to the following conclusions: PS enrichment to the ER-derived vesicles comes predominantly from the serine exchange enzyme, and VLCFA-PS and LCFA-PS species originate, respectively, from the serine exchange enzyme and PS synthase. A summary of PS synthesis and targeting from the ER to the EV in leek cells is presented in Fig. 5.

Why are VLCFA-containing PS species targeted to ER-derived structures? In plant cells, VLCFAs are primarily found

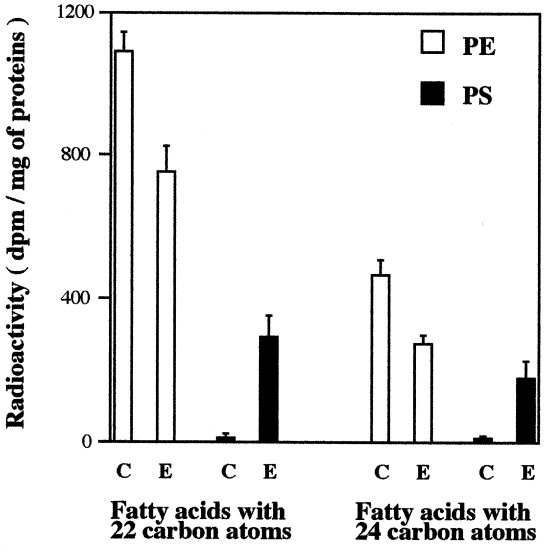


Fig. 4. VLCFA-PS synthesis from VLCFA-PE by the serine exchange enzyme activity. ER membranes were first incubated with [2-$^{14}$C]malonyl-CoA to synthesize VLCFAs and the VLCFA-PE. ER membranes were then incubated with unlabelled L-serine to synthesize VLCFA-PS (E for exchange enzyme) or not, or without incubation (C for controls). Lipid and FAME analyses were done as described in Section 2. After elongase activity, traces of [$^{14}$C]VLCFA-PS species were observed. Results are means of three independent experiments ± S.D.

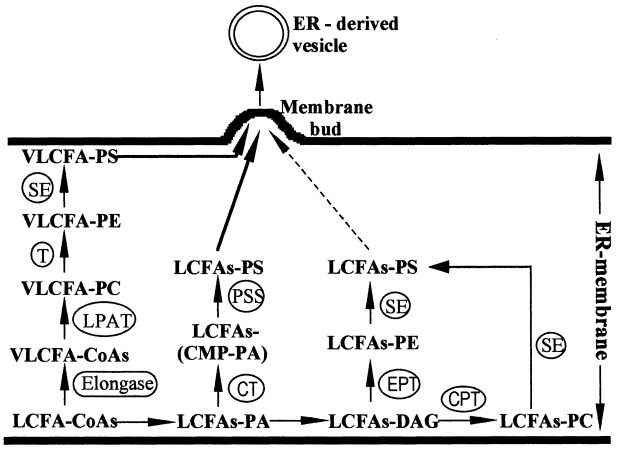


Fig. 5. Metabolic pathways involved in the synthesis of LCFA- and VLCFA-PS species which are targeted to ER-derived vesicles. After de novo synthesis in the plastids, the fatty acids are transferred to the ER as LCFA-CoAs. These are used by acyltransferases to produce LCFA-PA which is at the origin of all the LCFA-phospholipid species. LCFA-PS is formed from both PS synthase (PSS) and the serine exchange enzyme (SE). LCFA-CoAs can also be elongated to VLCFA-CoAs which are first incorporated into PC [17,28], an acyl donor for other lipid species [29]. VLCFA-PS are synthesized from VLCFA-PE by the serine exchange enzyme. Solid lines indicate PS species that are efficiently delivered to the EV. The dashed solid line indicates that the PS species from this pathway can be delivered but that its contribution must be low. LPAT: lyso PC acyltransferase; T: transacylase; CT: cytidylyltransferase; EPT: ethanolamine phosphotransferase; CPT: choline phosphotransferase; DAG: diacylglycerol.

in the phospholipids PC, PE and especially PS ([1,4]; this study) and sphingolipids [21]. In addition to the fact that PS accumulates at the plasma membrane [1] and comes from both the ER after intracellular transport [1,3] and local synthesis by the serine exchange enzyme [15], targeting PS with VLCFAs could have a role in the molecular mechanisms of the vesicular transport pathway.

It has recently been observed that overexpression of the FAE1 gene corresponding to the condensing enzyme of the fatty acid elongase complex in transgenic *Arabidopsis* plants leads to a strong accumulation of VLCFAs into glycerolipids and to dramatic alterations of plant morphology. One of the modifications was a high curvature of the thylakoid membranes [22,23]. Such considerations suggest that VLCFAs could have a role in the regulation of the curvature of membrane bilayers, by stabilizing highly curved membranes [24].

We now have a great deal of evidence that the ER membrane of plant cells is divided into areas with different functions [25]. It has also been demonstrated in animal cells that sphingolipids and cholesterol can be concentrated into membrane micro-domains called 'lipid rafts' [26]. Saturated VLCFA-PS, and other VLCFA-containing phospholipids, could favor a more ordered lipid phase where specific components are sorted, and the domain that has been formed could then be easily excluded (budded) from the ER membrane as suggested by the superlattice model [27].

*Acknowledgements:* This work was supported by the CNRS, the Université Victor Segalen Bordeaux 2 and the Conseil Régional d'Aquitaine. P.V. is the recipient of a doctoral fellowship from the Ministère de la Recherche, de la Technologie et de l'Enseignement Supérieur, France. We thank J. Pope for critically reading the English text.

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