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PLASMA MEMBRANE BIOGENESIS IN HIGHER PLANTS: *IN VIVO* TRANSFER OF LIPIDS TO THE PLASMA MEMBRANE*

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Key Word Index—*Allium porrum*; etiolated leek seedlings; very long chain fatty acids; plasma membrane biogenesis; intracellular lipid transfer.

Abstract—The intermembrane transfer of lipids and particularly of very long chain fatty acids (VLCFA) from a light membrane fraction (1.08–1.09 g/cm^3) to heavier membranes (1.16–1.18 g/cm^3) has been demonstrated in etiolated leek seedlings [Moreau *et al.* (1986) *Phytochemistry* **25**, 387.].

By means of biochemical markers (glucan synthetase II, IDPase, CDP-choline diglyceride transferase and NADPH cyt *c* reductase), it was shown that the plasma membrane is confined to the 1.16–1.18 g/cm^3 membrane fraction which is nevertheless heterogeneous. The plasma membrane was further purified in an aqueous two polymer phase system. In pulse-chase experiments, an increase of labelled lipids (PC, PE and neutral lipids) and of labelled fatty acids (C_{16} to C_{24}) in the plasma membrane was observed during the chase, whereas the total radioactivity of lipids of the microsomal pellet remained unchanged over the same period. The amounts of labelled C_{16}, C_{18} and VLCFA in the plasma membrane increased during the chase by two, three and four times respectively.

The biosynthesis of VLCFA was assayed *in vitro* in the purified plasma membrane and the other membranes after phase partition of the microsomal pellet. The purified plasma membrane was unable to elongate exogeneous substrates such as C_{18}-CoA and C_{20}-CoA or endogeneous substrates in the presence of ATP. Consequently, the results presented in this paper demonstrate clearly the transfer of VLCFA to the plasma membrane of a higher plant.

INTRODUCTION

The *in vitro* biosynthesis of very long chain fatty acids (VLCFA) by elongation of stearoyl-CoA has been shown to be localized in an ER-enriched membrane fraction of leek epidermis [1] and of etiolated maize coleoptiles [2]. This biosynthetic activity is three to four times higher in this fraction than in the plasmalemma-enriched membrane fraction. Paradoxically, VLCFA may represent up to 20% of the total content of fatty acids of the plasma membrane, whereas only a small amount of VLCFA is present in the ER-enriched membrane fraction. These results suggested an eventual transfer of the VLCFA from their site of synthesis (ER) to their sites of accumulation (plasmalemma and wax layer).

A methodology was devised to study the intermembrane transfer of lipids and more particularly VLCFA, by seven-day-old etiolated leek seedlings *in vivo* [3]. Thus by means of *in vivo* pulse chase experiments, followed by membrane subfractionation of microsomal pellets on a linear sucrose gradient (to give four membrane fractions called A, B, C and D), intermembrane transfer events of VLCFA between the light fraction A and the heavier ones were demonstrated [3]. However, these results did not establish that VLCFA were transfered to the plasma membrane.

The first part of the study presented in this paper shows that the heaviest membrane fraction (D) contains all the membrane vesicles originating from the plasma membrane, but is nevertheless relatively heterogeneous. In order to study the transfer of VLCFA to the plasmalemma further, the purification of this membrane has been undertaken, using a technique of phase partition in an aqueous two-polymer phase system [4]. In the second part of this paper, we present the isolation of the plasmalemma from etiolated leek seedlings and we describe for the first time, *in vivo* transfer events of lipids and fatty acids to the plasma membrane of a higher plant.

RESULTS

Characterization of the membrane fractions obtained on sucrose gradients

The microsomal pellet in terms of marker enzymes, is enriched in endomembranes and plasmalemma [3]. In order to study the origin of the different membrane structures found in the four membrane fractions A (1.08–1.09 g/cm^3), B (1.10–1.11 g/cm^3), C (1.13–1.14 g/cm^3) and D (1.16–1.18 g/cm^3), obtained after fractionation of the microsomal pellet on linear sucrose gradients (Fig. 1), various marker enzyme activities [CDP-choline diacylglycerol choline phosphotransferase and NADPH cyt c reductase as ER markers, inosine diphosphatase (IDPase) as a Golgi marker and glucan synthetase II (GS II) as a plasmalemma marker] were measured (Fig. 1).

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—*Abbreviations*: VLCFA, very long chain fatty acids; DTT, DL-dithiothreitol; PEG, poly-ethylene glycol; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

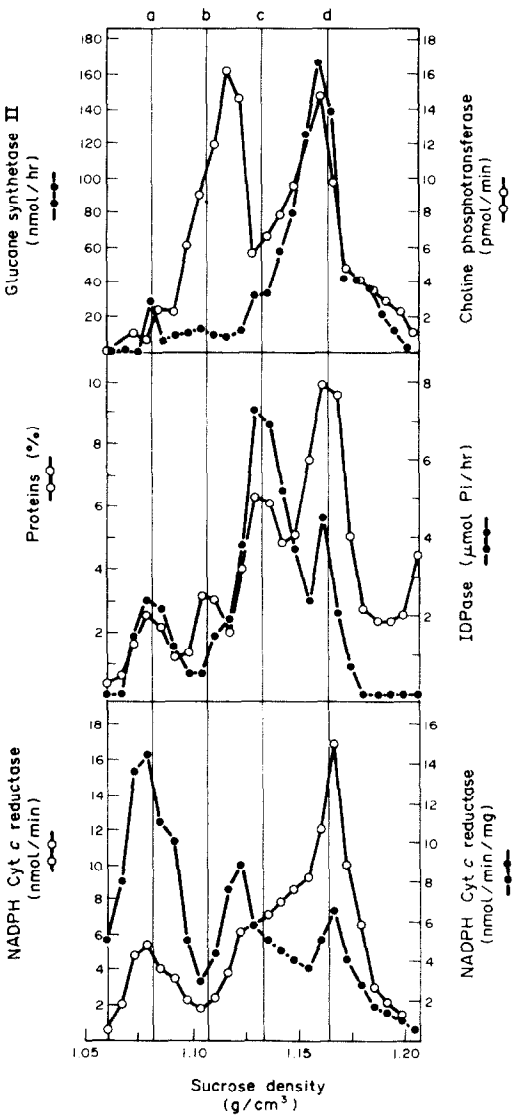

Fig. 1. Enzyme activity profiles after linear sucrose gradient centrifugation of the microsomal pellet. Membrane subfractionations were prepared according to the marker enzyme studies to be carried out (see Experimental). All results are expressed as total activities. NADPH cyt *c* reductase is also given per mg of proteins to illustrate the large difference between the distribution of the total activity and the activity per mg of protein for this marker.

The first maximum of the choline phosphotransferase activity is found in membranes sedimenting between 1.11 and 1.13 g/cm^3, corresponding to the interpeak region between fractions B (1.10–1.11 g/cm^3) and C (1.13–1.14 g/cm^3) (Fig.1). A second maximum is observed for membranes sedimenting with fraction D (1.16–1.18 g/cm^3). The highest specific activity is found in membranes sedimenting between 1.11 and 1.13 g/cm^3, in this example about 45 pmol/min/mg.

The results obtained for the NADPH cyt *c* reductase (Fig. 1) show that this activity is distributed along the entire gradient, with maxima observed at the level of fraction A (1.08–1.09 g/cm^3), at the interpeak between fractions B and C and in fraction D (1.16–1.18 g/cm^3). The highest specific activity of NADPH cyt *c* reductase (14.5 nmol/min/mg) is present in membranes of fraction A.

Choline phosphotransferase activity is largely considered to be an ER marker [5] but Montague and Ray [6] and Sauer and Robinson [7] have shown its presence in Golgi membranes in etiolated pea seedlings and maize roots respectively. Therefore, we must consider that this activity could also be associated with membrane vesicles originating from the Golgi apparatus.

The quantitative distribution of the IDPase activity (Fig. 1) shows a maximum in fraction A and another one at the level of fraction C (with a shoulder in fraction D). This activity has been found to be both non-particulate and confined to Golgi apparatus and secretory vesicles [5]. The non-particulate activity is generally attributed to non-specific phosphatases [5]. The activity associated with the Golgi apparatus is latent [5]. This latency (3 to 4 days at 4 °) has been correlated to the particulate activity [5] and, in many cases, has been correlated to Golgi structures [5]. However, data showing the presence of IDPase activity in ER and plasma membrane fractions were also obtained [8]. The IDPase activity measured on the density sucrose gradient was latent for three days at 4 °.

The membranes originating from the Golgi apparatus of higher plants are generally localized at a density of 1.12–1.15 g/cm^3 [5]. Sixty percent of the IDPase activity is found in the fraction C (1.13–1.14 g/cm^3), and therefore, it seems likely that fraction C contains the largest amount of membranes originating from the Golgi apparatus.

The last marker studied was GS II, this activity forms β 1–3 linkages and should lead to the synthesis of callose [5].

Figure 1 clearly shows that GS II activity is only present in fraction D (1.16–1.18 g/cm^3). As plasma membranes on sucrose gradients are localized between 1.13 and 1.18 g/cm^3 [5], this result indicates that all membrane vesicles originating from the plasma membrane are confined to this fraction.

Finally, Fig. 1 shows marker enzymes other than GS II that are present in the membrane fraction D, indicating the heterogeneity of the latter. Consequently, the *in vivo* transfer of VLCFA to fraction D [3] cannot be assimilated with a transfer to the plasma membrane uniquely. In order to demonstrate such transfers unambiguously, the isolation of the plasmalemma was undertaken by phase partition in an aqueous two polymer phase system [4].

Purification of the plasma membrane by phase partition

Phase partition allows the isolation of the plasma membrane by making use of its surface charge properties [10]. The plasmalemma has more affinity for the PEG-enriched upper phase, whereas other membranes remain confined to the dextran enriched lower phase and the interface. This method was found to be relatively quantitative since 85% of the total labelled lipids or 81% of the total proteins of the initial membranes were recovered in the upper and lower phases after such a partition (Moreau *et al.*, unpublished results).

Biochemical data showing the purification of the plasmalemma from the microsomal pellet is presented in

Table 1. The recovery of membrane proteins after phase partitioning shows that, despite a loss of membranes during phase partitioning, the proportion of membranes recovered in the different phases is reproducible (90.3% ±2.2 in the lower phase and 9.7% ±2.2 in the upper phase).

GS II specific activity (nmol/min/mg) is four times higher in the upper phase than in the lower phase, whereas CDP-choline diacylglycerol transferase and IDPase activities are largely present in the lower phase and at the interface (98.4 and 96% respectively). GS II activity in the upper phase is of the same order of magnitude as that found for purified plasma membranes from *A. sativa* and *B. oleracea* microsomes [11]. From the total GS II activities, we can estimate that the distribution of plasmalemma vesicles between the upper and lower phases is about 70% in the lower phase and only 30% in the upper phase.

However, 30% of GS II activity in the upper phase, compared to only 1.6 and 4% of choline phosphotransferase (an ER marker) and IDPase (a Golgi marker) activities, shows that the upper phase is enriched in plasma membrane vesicles.

Results of the lipid and fatty acid analyses of the various membrane fractions are shown in Table 1. The sterols and VLCFA contents of the membranes and the degree of saturation of the fatty acids of these membranes also indicated the enrichment of the plasmalemma in the upper phase.

Indeed, plasmalemma vesicles isolated from maize coleoptiles contain more sterols [12] and VLCFA [13] than do other membranes. Moreover, the amounts of saturated fatty acids are higher in the plasma membrane than in endoplasmic reticulum or intracellular organelles [13]. In the same manner, plasma membrane vesicles isolated from *Allium porrum* epidermis cells contain more VLCFA than any other membrane [13]. Finally, the phospholipid/galactolipid ratio shows the low contamination if any of the plasmalemma enriched fraction by plastid membranes, i.e. membranes with a high galactolipid content [14].

In order to estimate the eventual contamination of the plasma membrane enriched fraction by tonoplast, ATPase activity was assayed according to Sze [15] (Table 2). Firstly, stimulation of the ATPase activity by K^+ is greater in the upper phase (+169%) than in the lower phase (+30%). This stimulation by K^+ is a feature of plasma membranes [16, 17] and is considered to be a marker for this membrane.

The inhibition of the K^+-stimulated ATPase activity by vanadate is 93.3% in the upper phase and 49.5% in the lower phase. The inhibition of the ATPase activity by vanadate being specific to the plasmalemma ATPase [15], an inhibition of 93.3% in the upper phase demonstrates a great enrichment of plasma membrane vesicles.

ATPase activity assayed in the presence of nitrate is only inhibited by 9.9% in the upper phase and by 46.3% in the lower phase. Since, only tonoplast and mitochondrial ATPases [15] are affected by this inhibition, the plasma membrane is only weakly contaminated by tonoplast and mitochondrial membranes.

The degree of purification of the plasma membrane in the upper phase, according to the level of choline phosphotransferase activity (Table 1), needs comment. If we assume that the microsomal preparation contains 15–20 times more endoplasmic reticulum than plasmalemma, as estimated from preliminary analyses, then the 1.6% (Table 1) corresponds to a contamination of 25–30%. The latter is certainly overestimated because the degree of inhibition of ATPase activity by vanadate in the upper phase (Table 2) shows unambiguously a high enrichment in plasma membrane vesicles. Furthermore, we found no

Table 1. Biochemical data showing the purification of the plasma membrane in the upper phase after phase partition of the microsomal pellet

	Lower phase	Upper phase
Glucan-synthetase II (nmol/min/mg prot.), $N=3$	2.6±1.4 (70%)	10.6±4.1 (30%)
Choline phosphotransferase (pmol/min/mg prot.), $N=2$	13.2(98.4%)	2.1(1.6%)
Inosine diphosphatase (μmol/hr/mg prot.)	3.4(96%)	1.3(4%)
Phospholipids	1.9	11.5
Galactolipids (μg/μg)		
Sterols(%μg/μg lipids) $N=4$	7.4±0.2	16.6±2.8
VLCFA (% weight/weight of total fatty acids)	2.1	6.3
Saturated fatty acids		
Unsaturated fatty acids (w/w)	0.35	0.9
Proteins (%) $N=8$	90.3±2.2	9.7±2.2

For marker enzymes, the data given in parentheses show the distribution of each total activity between the lower and the upper phases. N values give the number of experiments that were performed, in some cases.

VLCFA synthesis in the plasma membrane fraction (Table 3) and it is known that the ER is an important site of biosynthesis of these molecules [1, 18].

From these results, we can conclude that the plasma membrane fraction is highly enriched and suitable for the study of lipid and VLCFA transfer to the plasma membrane.

In vivo transfers of lipids and fatty acids to the plasmalemma

After pulse-chase experiments carried out as described before [3] and in the experimental section, the purified plasma membrane fraction was prepared. Lipid and fatty acid analyses were then performed according to the Experimental section.

The radioactivity of the total lipids of the plasma membrane as a function of the chase time following labelling for 1 or 2 hr is shown in Fig. 2. The total lipid label of the crude microsomes is also indicated in the legend of the figure. In the two experiments, an increase of the radioactivity content of the total lipids of the plasma membrane was observed during the chase, whereas the radioactivity content of total lipids in the microsomal pellets remained unchanged during the same time. After a labelling period of 2 hr (Fig. 2A), the amount of labelled lipids in the plasma membrane fraction represents, 2.7% of the lipid label of the crude microsomes. Then, after a chase of 120 min (Fig. 2A), this amount reaches 6.3%. After a labelling for 1 hr (Fig. 2B), the amount of labelled lipids in the plasma membrane fraction represents only 1.9% of the lipid label of the crude microsomes. Following a chase of 30 min, this amount is about 4.6% of the lipid label of the crude microsomes.

The increase of labelled lipids in the plasma membrane fraction shows that a transfer of lipids to this membrane is likely to have occurred *in vivo*. In the case of a labelling period of 1 hr, an increase of the radioactivity of the plasma membrane was first observed and then a decrease after a 1 hr chase period was found. This decrease could be explained by the fact that part of the lipids incorporated in the plasma membrane can be then transferred to the wax layer [18].

This decrease is not observed after a labelling period of 2 hr, probably because the amount of lipids that are transferred to the plasma membrane is higher than the amount of lipids transferred from the plasmalemma to the wax layer. Moreover, the pool of lipids that can be transferred to the plasma membrane is greater after a 2 hr labelling period than after a 1 hr labelling period.

Lipid analysis (Figs 3A and 3B) revealed that the transferred lipids were essentially the neutral lipids (including sterols), PC and PE. These lipids being constitutive of the plasma membrane [19]. Thus our results demonstrate the *in vivo* transfer of lipids to the plasmalemma. The results obtained for the VLCFA after a 2 hr labelling period are presented in Table 4. The amount of labelled VLCFA in the crude microsomes remains constant (18 300 cpm $\pm$ 550) during the chase period. By contrast, the label found in the VLCFA of the plasma membrane fraction is greatly increased after a 120 min chase period. After the labelling period, the amount of labelled VLCFA in the plasma membrane fraction represent 10.2% of the total radioactivity of the VLCFA of the crude microsomes. Then, after a chase of 120 min, this amount reaches 31.5%. According to the GS II activity, the plasma membrane fraction obtained in the upper

Table 2. ATPase activities in the upper and lower phases obtained after phase partition of the microsomal pellet

Experimental conditions	Upper phase	Lower phase
$MgSO_4$ (3 mM)	14.3(27.5)	73.0(11.8)
+KCL (50 mM)	38.5(74.0)	95.0(15.3)
+KCL (50 mM)		
+Na_3VO_4 (50 μM)	2.6(5.0)	48.0(7.7)
+KCL (50 mM)		
+NO_3 (10 mM)	34.7(66.7)	51.0(8.2)

The results are expressed in nmol Pi formed/min. Data given in parentheses represent these activities per mg of proteins.

Table 3. Elongation activities in the lower and upper phases obtained after phase partition of the microsomal pellet

	A		B		C	
Exogeneous substrate	None		C_{18}-CoA		C_{20}-CoA	
ATP	+		−		−	
Phases	lower	upper	lower	upper	lower	upper
Total activities (nmol/hr)	54	0.08	17.1	0.11	3.4	0.02
Specific activities (nmol/hr/mg)	12	0.16	3.8	0.22	0.75	0.04

Forty-five μg of protein of the lower phase or 30 μg of protein of the upper phase were incubated 1 hr at 30° in the presence of:

—A: [2-^{14}C]malonyl-CoA(17 μM), NADH and NADPH (0.5 mM), ATP (1 mM), $MgCl_2$ (1 mM), dithiothreitol (2 mM), HEPES buffer 0.08 M, pH 7.5 (final volume: 0.1 ml).

—B and C: same conditions as in A but without ATP and in the presence of C_{18}-CoA or C_{20}-CoA 9 μM respectively.

Activities are expressed as nmol of [2-^{14}C]malonyl-CoA incorporated.

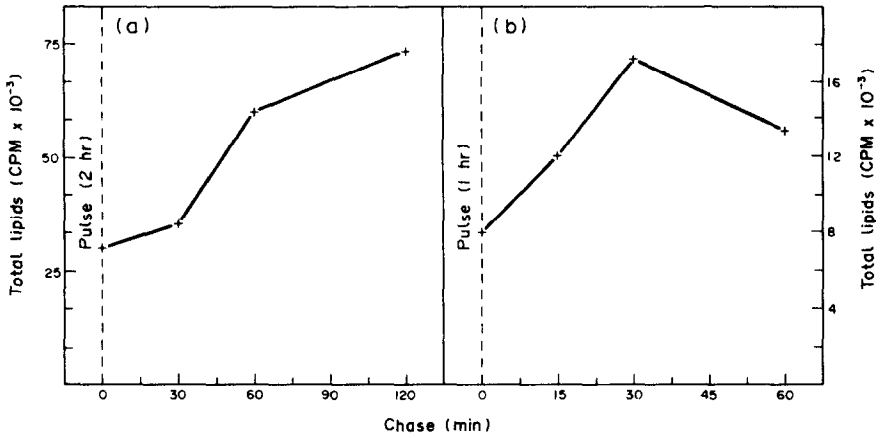

Fig. 2. Radioactivity in the total lipids of the plasma membrane during the chase following a 2 hr labelling period (a) or a one-hour labelling period (b). All experimental procedures were performed as indicated in Experimental. Results are expressed as cpm per mg of protein, which was determined by Bradford's method [44]. The total label of the crude microsomes was $1\,180 \pm 70$ cpm $\times 10^{-3}$ in experiment (a) and 380 ± 36 cpm $\times 10^{-3}$ in experiment (b).

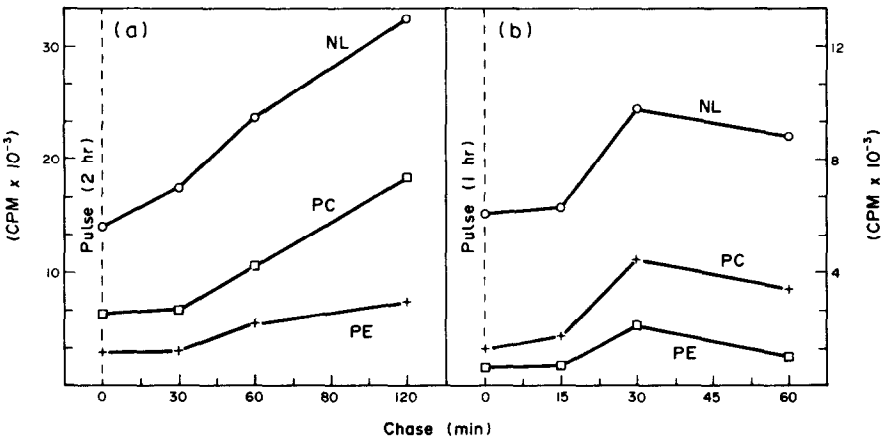

Fig. 3. Radioactivity content of the neutral lipids (NL), PC and PE in the plasma membrane as a function of the chase time following a two-hour labelling period (a) or a one-hour labelling period (b). Same experimental conditions as in Fig. 2.

Table 4. Radioactivities of the VLCFA of the microsomes and of the plasma membrane fraction as a function of the chase time following a 2 hr labelling period

Chase time (min)	Microsomes	Plasma membrane
0	17500	1800(10.2%)
30	18200	1900(10.4%)
60	19000	3600(18.4%)
120	18700	5900(31.5%)

phase (Table 1) represents about 30% of the total plasma membrane. Consequently, after a chase of 120 min, 31.5% of the labelled VLCFA are present in 30% of the plasma membrane. These results could suggest that the VLCFA were integrally transferred to the plasma membrane during the chase. Lastly, Fig. 4 presents the radioactivity of C_{16} and C_{18} fatty acids and of VLCFA in the plasma membrane as a function of the chase time. The results are expressed for each fatty acid with respect to the radioactivity determined for the labelling time, which is taken as being equal to 100.

Figure 4 shows an enrichment of the plasma membrane in VLCFA and to a lesser extent in C_{18} fatty acids with respect to C_{16} fatty acids. The results obtained for the VLCFA raise the following question: is the increase in the radioactivity of the VLCFA in the plasmalemma due only to transfer events or is it also partially due to a local biosynthesis of these molecules in this membrane? In leek epidermis cells, Cassagne and Lessire [1] have found the highest elongation activity in an ER enriched membrane fraction but found also some activity in a membrane fraction partly enriched in plasma membranes.

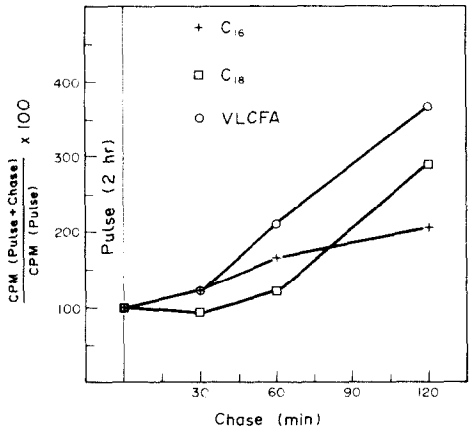

Fig. 4. Radioactivity of the various fatty acids of the plasma membrane as a function of the chase time following a 2 hr labelling period. Same experimental conditions as in Figs 2a and 3a.

To answer this question, VLCFA synthesis was assayed in the plasma membrane enriched fraction (upper phase) and in the lower phase after phase partition of the microsomal pellet. Table 3 presents the results of this study. In the upper phase, the ATP-dependent elongation activity represents 0.15% of the total activity found in the lower phase, while the C_{18}-CoA and C_{20}-CoA elongase activities are only 0.6% of those observed for the lower phase. Table 3 also shows that specific activities (per mg of protein) in the lower phase are respectively 75, 17 and 19 times higher than those of the upper phase for the ATP-dependent elongase and the C_{18}-CoA and C_{20}-CoA elongases. Moreover, the various elongase activities of the lower phase are of the same order of magnitude as those found in the microsomal pellet [20], showing that no loss of activity has occured during the phase partition. It has been shown that PEG and Dextran have no inhibitory effect on elongase activities (Lessire *et al.*, unpublished results). So, the absence of any elongation activity in the purified plasma membrane demonstrates that this membrane is unable to synthesize VLCFA. Consequently, the results shown in Table 4 and Fig. 4 clearly demonstrate that VLCFA have been transferred to the plasma membrane *in vivo*.

DISCUSSION

The results presented in this paper demonstrate for the first time the transfer of lipids, and particularly of VLCFA, to the plasma membrane of a higher plant *in vivo*. These transfers have been demonstrated by: (i) the preparation of a highly enriched plasma membrane fraction by means of phase partition in an aqueous two-polymer phase system. (ii) Studies of CDP-choline diacylglycerol transferase activity and of the various elongating activities which have shown that the plasma membrane is not able to synthesize PC and VLCFA. (iii) *In vivo* pulse-chase experiments which allowed us to demonstrate the existence of the transfer of lipids and fatty acids.

The question which is raised now is how are the lipids and VLCFA transferred to the plasma membrane? It is well known that the Golgi apparatus has a central position in the membrane flow concept in animal cells [21, 22] and in plant cells [23, 24]. It has been shown that the Golgi complex is involved in the transport of proteins to the plasma membrane both in animal cells [21, 25] and plant cells [26–30]. In animal cells, Pagano *et al.* [9] have also proposed the involvement of this organelle in the intracellular movement of lipids. Monensin and nigericin are ionophores known to disturb the vesicular traffic at the level of the Golgi apparatus [31–35] and have been used to demonstrate the involvement of this organelle in the intracellular transport of proteins [26, 36–38] and of lipids in animal cells [9, 39].

The use of such a drug in studies of the transfer of lipids and fatty acids to the plasma membrane should provide informations concerning the eventual involvement of the Golgi apparatus in higher plants.

Previous results [3] have shown an intermembrane transfer of VLCFA from fraction A to the heavier fractions and particularly to fraction D. The presence of NADPH cyt *c* reductase and IDPase activities in fraction A suggests that this fraction is chiefly constituted by vesicles originating from the ER and the Golgi apparatus. The fact that these marker enzyme activities are lower in fraction A than in other membrane fractions (fraction D for NADPH cyt *c* reductase and fraction C for IDPase) indicates that the vesicles in this fraction only represent a small amount of the initial membranes of the ER and Golgi apparatus. Moreover, fraction A is not the site of synthesis of PC, as shown by the distribution of the choline phosphotransferase activity (Fig. 1) and not the site of synthesis of VLCFA. Therefore, some of the vesicles present in fraction A could originate from particular domains of the ER and/or of the Golgi apparatus and could also be intermediary or transfer vesicles, the latter possiblity being supported by the reported intermembrane transfer events [3]. The participation of the membranes of fraction A in these transfer events will be further investigated.

The determination of the intracellular localization of the various elongating activities, and also the distribution of the acyl-CoAs transacylases, is an absolute requirement to establish the form in which the fatty acids are transferred. For example, it has been demonstrated that VLCFA are synthesized as acyl-CoAs by C_{18}-CoA and C_{20}-CoA elongases [40], so the presence or the absence of acyl-CoA transacylases in the plasma membrane will indicate whether, or not, fatty acids (including VLCFA) could be transferred to this membrane as acyl-CoAs.

Fatty acid binding proteins, which also bind acyl-CoAs [41], could be involved in such transfers. Such studies lead us to consider the possible role played by phospholipid transfer proteins in this process. Indeed, it is already established that, *in vitro*, phospholipid transfer proteins are able to transport lipids between donor and acceptor membranes [42, 43] and, hence, could also be involved in the transfer of lipids to the plasma membrane.

In conclusion, etiolated leek seedlings should allow to investigate *in vivo* the eventual role played by membrane traffic (via the Golgi apparatus or otherwise) and by specialized proteins in the transport of lipids to the plasma membrane of a higher plant.

EXPERIMENTAL

Plant material. Leek (*Allium porrum L.*) seeds, stored overnight at 4°, were sterilized with $NaClO_4$ in the presence of

Triton X-100 for 5 min and then washed with dist. H_2O. They were then grown for 7 days in the dark, at 25°, on the following growth medium: 5 g agar-agar, 900 ml of dist. H_2O and 100 ml of a nutritive soln containing 7.5 g/l KCl, 6 g/l $NaNO_3$, 4.5 g/l $MgSO_4$, 0.95 g/l $CaCl_2$ and 1.25 g/l NaH_2PO_4.

Membrane subfractionation. The seedlings were homogenized in various buffers appropriate to the marker enzymes studies to be carried out. The homogenate was centrifuged for 5 min at 1000 g. The supernatant so obtained was centrifuged for 15 min at 12 000 g and the resulting supernatant was spun for 60 min at 150 000 g. The microsomal pellet was loaded onto a linear sucrose gradient (1.04–1.22 g/cm^3) which was centrifuged for 18 hr at 130 000 g. The gradient was then fractionated with a Gilson collector (sampling being 1 ml per fraction) for a gradient of 35 ml. Proteins were estimated by Bradford's method [44]. Whatever the various buffer/pH conditions used, the profiles of proteins were reproducible [3].

Assay of marker enzymes

NADPH cytochrome c reductase. Membrane fractionation was effected in 0.05 M Tris–HCl buffer, pH 7.5, and enzymatic assays were performed using the method of ref. [45].

Glucan synthetase II. Membrane fractionation was effected in 0.08 M HEPES buffer, pH 7.8. The activity was measured by the method of ref. [46] modified according to ref. [47]. UDP [U-^{14}C] glucose (304 Ci/mol) was used as the radioactive substrate.

Choline phosphotransferase. Membrane fractionation was performed in 0.08 M HEPES buffer, pH 7.8, containing DTT (4 mM). The highest activity in microsomes was found using $MnSO_4$ (4.5 mM) and was 4 times higher than in the presence of $MgCl_2$ (10 mM). Moreover, no stimulation by $MgCl_2$ was obtained in the presence of $MnSO_4$. For the assays, 100 μl of the membranes (10–100 μg of proteins according to the membrane source) were incubated for 5 min at 30° with 20 μl of $MnSO_4$ (4.5 mM). Then 0.5 μCi of CDP-[^{14}C]choline (42 Ci/mol) were added and the incubation was continued for 30 min at 30°.

Reactions were stopped with 1 ml $CHCl_3$ and by 1 min at 100°. Lipids were extracted as described by Heape *et al.* [48] and the label in phosphatidyl-choline was determined in a liquid scintillation counter.

Inosine diphosphatase. Membrane fractionation was performed in 0.05 M Tris–MES buffer, pH 7.5. Latency was removed after 3 days at 4°. To prevent microbial contamination during storage at 4°, membranes were prepared and stored in the presence of 0.1% NaN_3. For each assay, 50 μl of membrane proteins were incubated for 60 min at 30° in the presence of $MgSO_4$ (3 mM), KCl (50 mM) and 1.65 mg of IDP (4 μmol). Pi release was estimated according to ref. [49].

Adenosine triphosphatase. Membrane fractionation was realized in 0.05 M Tris–HCl buffer, pH 7.5. Plasma membrane isolation was performed as described below. The activity was measured according to Lessire [13] and the specific inhibitory effects of Na_3VO_4 and nitrate ions on ATPase activities of plasmalemma and tonoplast were determined according to Sze [15].

Isolation of plasmalemma. Seedling homogenization and membrane subfractionation were performed in 0.08 M HEPES buffer, pH 7.8. Membranes of the microsomal pellet (corresponding to 1 mg of proteins) were then homogenized in 25 ml of the following mixture: dextran T500 5.6% (w/w), poly-ethylene glycol (PEG 4 000) 5.6% (w/w) in 0.5 M sorbitol buffer, pH 7.8, containing KH_2PO_4 (10 mM) and NaCl (40 mM).

After 10 min at 1000 g, the PEG enriched upper phase was recovered without disturbing the interface which was recovered with the dextran enriched lower phase. Membranes were then recovered after centrifugation at 250 000 g for 90 min and resuspended in the appropriate buffer.

Lipid analysis. Lipid extraction and analysis were performed according to Heape *et al.* [48]. Quantitation of lipids was carried out from calibration curves that were established using standard lipids [50].

Fatty acid analysis. Two types of acid analysis were performed. The first used a 10% CP-SIL 58 column (1/8" × 2 m) on WHP (100–200 mesh size), and allowed the separation of mono- and polyunsaturated fatty acids from saturated fatty acids. The second used a capillary column and allowed a better quantitation of saturated fatty acids having up to 30 carbon atoms. For the two types of analysis, the identification of the various fatty acids was performed by comparison with methyl ester standards.

In vivo *transfer of lipids and fatty acids to the plasmalemma. In vivo* [1-^{14}C]acetate (55 Ci/mol) incorporations and pulse-chase experiments were performed as described [3]. Membrane fractionation and plasma membrane isolation were carried out as indicated above. Lipid and fatty acid radioactivities were then determined after analysis of lipids according to ref. [48] and analysis of fatty acids as described previously [3].

Assay for VLCFA synthesis in vitro. Plasma membrane isolation was performed as indicated above. Membrane vesicles were resuspended in 0.08 M HEPES buffer, pH 7.5. Proteins of the lower phase (45 μg) or the upper phase (30 μg) were incubated 1 hr at 30° in different media depending on the activity tested.

C_{18}-CoA and C_{20}-CoA elongase activities were measured in the presence of 1 μCi [2-^{14}C]malonyl-CoA (57 Ci-mol) 17 μM, C_{18}-CoA or C_{20}-CoA (9 μM), NADH and NADPH (0.5 mM), $MgCl_2$ (1 mM), DTT (2 mM) for a final vol of 0.1 ml.

ATP-dependent elongase activity was assayed without exogenous fatty acyl-CoA, in the presence of ATP (1 mM) and 1 μCi of [2-^{14}C]malonyl-CoA (17 μM).

Radioactivities of VLCFA were determined according to ref. [20].

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