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LIPID METABOLISM DURING GERMINATION OF TOBACCO POLLEN

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Key Word Index—*Nicotiana sylvestris*; Solanaceae; pollen germination; lipid composition; acetate incorporation; phosphate incorporation.

Abstract—The variation of tobacco pollen lipids was studied during a germination time of four hr. During this time pollen grains went through a lag phase of 30 min after which pollen tube growth started. After four hr this growth had extended the area of the plasmamembrane by factors of five to 10. The major lipids were neutral lipids followed by phospholipids, whereas glycolipids represented only a minor fraction. The increase in membrane surface during pollen tube growth was not reflected by a similar increase in lipid content, which increased only slightly during the lag phase. Labelling with radioactive acetate showed a continuous incorporation of precursor into all lipid components from the beginning of germination, whereas labelling by radioactive phosphate commenced later. The data are discussed in terms of membrane assembly and turnover during pollen germination.

INTRODUCTION

Pollen germination is usually triggered by hydration of the dry pollen grain. After a lag phase the pollen tube protrudes and elongates rapidly [1]. The tube growth is accompanied by an intense tube wall synthesis [2] which rapidly increases the tube surface reaching multiples of its original area within short periods of growth [3]. Concomitant with this rapid surface extension, the plasmamembrane and the vacuolar membranes are enlarged. Accordingly, a high rate of membrane lipid synthesis is expected to parallel these processes. Therefore, the growing pollen tube should be useful for studying the metabolism of lipids from the plasma membrane and endomembrane system without too much interference from plastid lipid turnover which dominates in photosynthetically active cells. Since only a few investigations have been carried out on this subject [4–7], we started to analyse these events in terms of lipid metabolism. We studied the composition of the lipid mixture from ungerminated pollen grains and its variation during early times of tube growth and carried out experiments on the lipid turnover by using labelled acetate and phosphate.

RESULTS

Variation of lipid pattern

In a first series of experiments the variation of lipid quantities as a function of germination time was studied. The time interval between pollen hydration and microscopically visible tube growth initiation was *ca* 30 min and will be referred to as the lag phase. From 100 mg of ungerminated pollen grains a lipophilic fraction was extracted with chloroform–methanol which was separated by CC into neutral lipids (6 mg), glycolipids (0.2 mg) and phospholipids (2.5 mg). Throughout the germination period of 4 hr the quantity and composition of the total lipid mixture varies only slightly (Fig. 1a), particularly when regarding the period after the lag phase.

The predominant component in the neutral lipid fraction was triacylglycerol (TG) (2 mg), although TLC showed the presence of many other compounds, from which hydrocarbons, steryl esters (SE) and free sterols were tentatively identified by cochromatography but not quantified. During germination neutral lipids and TG decrease continuously, whereas glyco- and phospholipids display different patterns.

Common to extraplastidic glyco- and phospholipids is a biphasic behaviour [cerebroside (CE), sterol glycoside (SG) and acylated sterol glycoside (ASG) in Fig. 1b; phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI) in Fig. 1c]. During the lag phase their quantities are significantly increased. In the case of CE, the original concentration is more than doubled. With tube growth initiation after 30 min this elevated level either remains nearly constant (CE, SG) or decreases again, in the case of PC and PI even below quantities present in ungerminated pollen grains.

Plastid lipids [monogalactosyl diacylglycerol (MGD), digalactosyl diacylglycerol (DGD) and part of phosphatidyl glycerol (PG)] are less affected by hydration and tube growth, as their quantities remain largely constant or increase slightly (Fig. 1b, c). The mitochondrial lipid diphosphatidyl glycerol (DPG) (Fig. 1c) seems to follow a biphasic pattern similar to the other phospholipids. Since all the major phospholipids were subject to a similar variation, their proportions did not change significantly during four hr of germination. Therefore, this fraction was characteristically composed of PC (55%), PE (23%) and PI (19%), whereas DPG (2%) and PG (1%) represent minor quantities.

The fatty acid composition of the phospho- and glyco-

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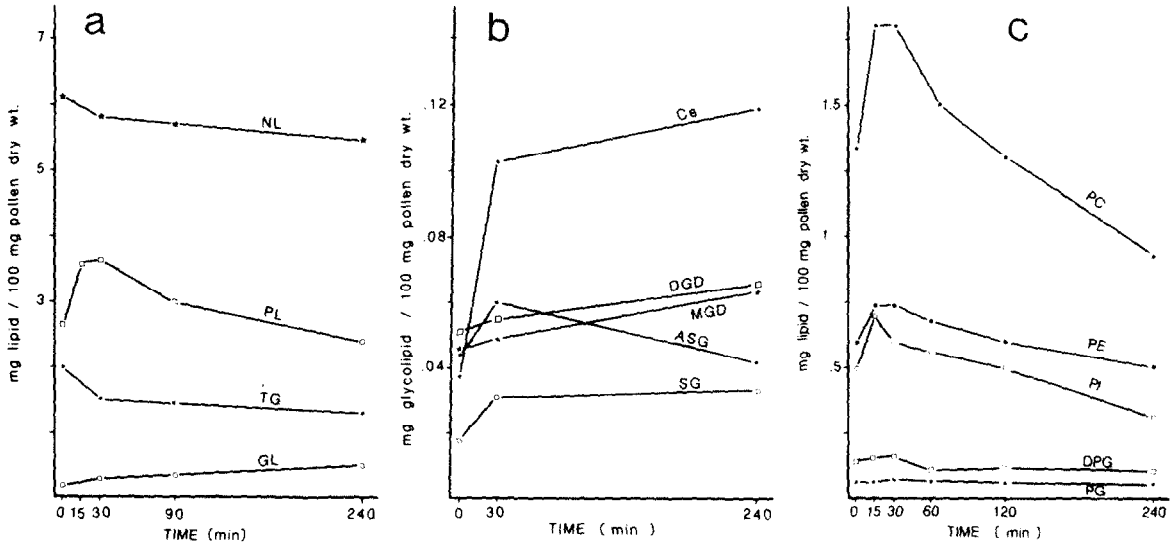

Fig. 1. Lipid changes during germination of *Nicotiana sylvestris* pollen. Pollen tubes start protruding after 30 min of incubation. (a) Lipid classes: NL = neutral lipids, PL = phospholipids, TG = triacylglycerols, GL = glycolipids. (b) Glycolipid fraction: CE = cerebroside, DGD = digalactosyl diacylglycerol, MGD = monogalactosyl diacylglycerol, ASG = acylated sterol glycoside, SG = sterol glycoside. (c) Phospholipid fraction: PC, PE, PG, PI = phosphatidyl choline, -ethanolamine, -glycerol, -inositol, DPG = diphosphatidyl glycerol.

lipids (Table 1) is in agreement with data from non-green tissues of tobacco [8,9]. MGD does not contain hexadecatrienoic acid and PG lacks *trans*-3-hexadecenoic acid. In most lipids, the major fatty acids are palmitic, linoleic and linolenic acid.

Labelling experiments

For lipid labelling, pollen grains were germinated in the presence of [^{14}C] acetate or [^{32}P] phosphate and extracted after various times of continued incubation in the presence of the radioactive substrate. The total lipid extract was separated into lipid classes before determination of radioactivity in individual components.

Uptake of acetate into lipids started immediately upon hydration without a lag phase and continued at a nearly constant rate throughout the two hr of investigation. The proportion of label in neutral lipids (65%), phospholipids (26%) and glycolipids (3%) reflected their proportion on a weight basis (Fig. 2a). The kinetics of labelling of individual components in the neutral fraction (Fig. 2b) demonstrates the constant acetate incorporation into TG, sterols, SE and diacylglycerols (which were not detected in unlabelled form). Individual glycolipids were also labelled at a contstant rate (Fig. 2c). DGD showed the highest incorporation in contrast to its proportion on a weight basis. A similarly constant labelling rate was observed for phospholipids, amongst which PC carried most of the label (85%), whereas PE was only little labelled (Fig. 2d).

Apart from the fact that usually only one phosphate group is incorporated per lipid molecule, phosphate labelling differed from acetate incorporation in two ways. Despite its higher sp. act., far less radioactivity was recovered in the lipid fraction (Fig. 3a). In addition, this uptake showed a biphasic behaviour: a slow incorporation up to 60 min followed by a time of significantly increased labelling (Fig. 3b). PE and PC were labelled to the same extent in contrast to acetate labelling and mass proportions.

DISCUSSION

The extraction of pollen grains and tubes with chloroform–methanol yields a complex mixture of compounds which were separated by CC into three fractions: neutral lipids, glycolipids and phospholipids. The identification of individual components in these fractions is not complete, since in each group several compounds separated by TLC were not identified. In the neutral fraction, TG was the major component, but also sterols and SE were tentatively identified by TLC in agreement with previous results from pollen of other species [10–13]. Increasing evidence indicates that free sterols are concentrated in the plasmamembrane, whereas the precise localization of SE is unknown [14]. The glycolipid fraction contained several unidentified compounds. Those identified originate from plastids (MGD, DGD) and tonoplast, plasmamembrane and other extraplastidic membranes (CE, SG, ASG). The low content of galactolipids reflects the small number of plastids present in these cells. Apart from two unknown, but minor components all phospholipids were identified. This fraction was dominated by PC and PE as usually observed in plant tissues [15]. The amount of DPG was relatively high. This phospholipid is restricted to mitochondria [16] and in fact, numerous mitochondria were seen in electron micrographs from tobacco pollen tubes [17, 18]. The fatty acid composition of the various lipids (Table 1) did not differ from data obtained from other non-green tissues [8, 9, 16]. All lipids contained appreciable proportions of linoleic and linolenic acid, whereas hardly any unsaturated fatty acids

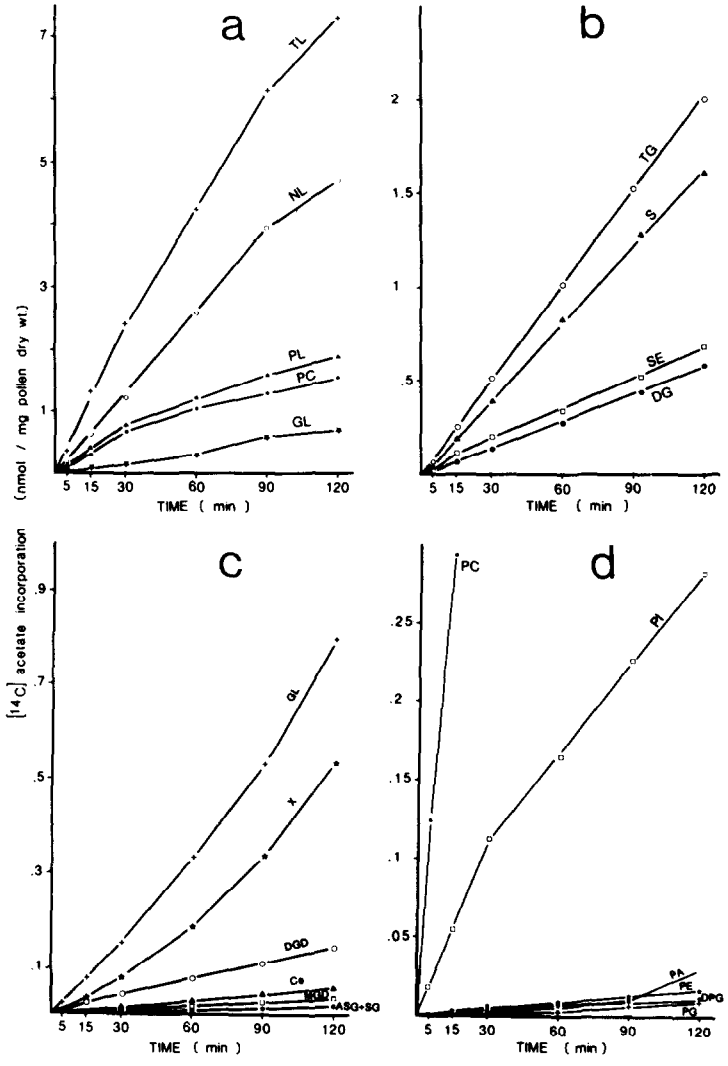

Fig. 2. Incorporation of [1-^{14}C] acetate into lipids during germination of *Nicotiana sylvestris* pollen. Only those abbreviations not used in Fig. 1 are explained here. (a) Lipid classes: TL = total lipids. (b) Neutral lipids: S = sterols, SE = sterol esters, DG = diacylglycerols. (c) Glycolipids: most of the label in this fraction is not accounted for by identified compounds. (d) Phospholipids: PA = phosphatidic acid.

have been detected in phospholipids from *Pinus* pollen [12].

Upon hydration and germination the extraplastidic glyco- and phospholipids change characteristically in quantity. During the lag phase of 30 min these components show a net increase. This is difficult to understand, since an equivalent increase in membrane area does not commence before the initiation of tube growth, i.e. after the end of the lag phase. Furthermore, after the lag phase the quantities of extraplastidic glycolipids such as SG and CE remain at an elevated level, whereas phospholipids decrease again, in some cases even below quantities present in the pollen grain. In contrast to these membrane lipids, TG are subject to a continuous decrease in quantity without difference between lag phase and subsequent period of tube growth.

The decrease of phospholipid levels may be partially explained by the following observations. Microscopic evaluations have shown that *ca* 20–30% of tobacco pollen grains do not survive the hydration process and therefore fail to germinate. It is well known that hydration is a most critical step in which preexisting membrane fragments are reassembled into functional membranes required for germination and tube growth [19]. In addition, a recent investigation has demonstrated the presence of phospholipid degrading enzymes in pollen grain homogenates [7]. Therefore, part of the disappearance of phospholipids after hydration may be ascribed to the degradation of phospholipids in hydrated, but ungerminated pollen grains.

The labelling experiments reveal patterns which are similar for all compounds, but differ from the kinetics observed with unlabelled components. With acetate as precursor all lipids take up radioactivity at a constant rate throughout the lag phase and the subsequent time. This indicates a continuous synthesis of these compo-

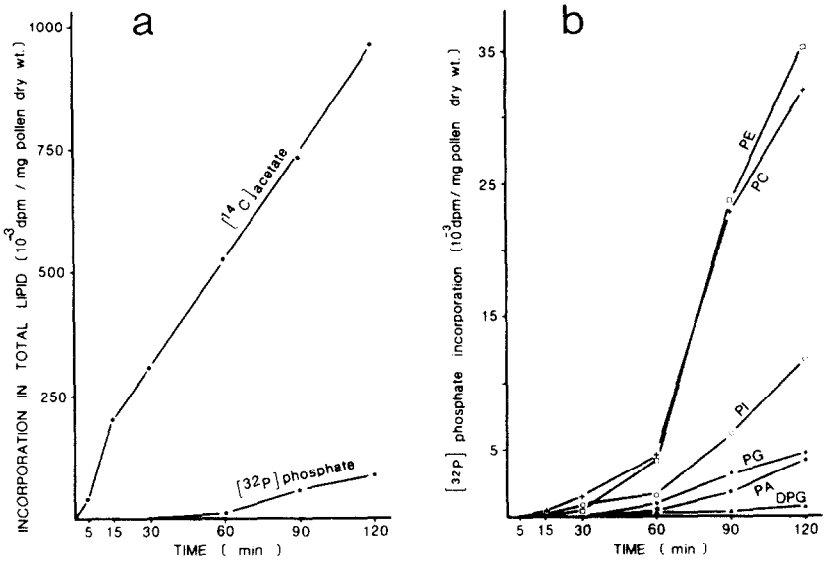

Fig. 3. Incorporation of [^{32}P] phosphoric acid into lipids during germination of *Nicotiana sylvestris* pollen. (a) Total lipids: for comparison, data from a parallel acetate labelling experiment are shown. (b) Phospholipid labelling.

Table 1. Fatty acid composition (wt %) of lipids from *Nicotiana sylvestris* pollen grains

Lipids	Fatty acids						
	16:0	16:1	18:0	18:1	18:2	18:3	Others
TG	21	—	2	8	46	19	4
SE	42	—	1	7	40	10	—
ASG	48	7	7	15	18	5	—
MGD	5	1	1	5	52	36	—
DGD	25	4	4	7	35	24	—
DPG	5	—	—	6	65	23	—
PG	44	—	1	2	37	16	—
PI	47	—	1	1	28	23	—
PE	30	—	—	2	54	14	—
PC	27	—	—	2	55	16	—

nents and does not reflect the biphasic behaviour observed with unlabelled lipids (cf. Figs 1 and 2). Also TG show these kinetics indicating synthesis even in this fraction which would have been anticipated to be used up during germination. But in fact, of all components studied, TG represent the fraction most heavily labelled.

The incorporation of phosphate into phospholipids differed from acetate labelling because of its biphasic nature. The low labelling at the beginning may indicate a limited uptake into the cells or the presence of a large intracellular supply of phosphate. It is known that pollen grains contain inositol hexaphosphate which is metabolized during the first hours of germination [20, 21]. Therefore, acetate is a more suitable precursor to study the early phase of lipid metabolism after pollen hydration.

The differences between the quantitative changes of lipid patterns and the constant incorporation of radioactivity into all lipid classes are difficult to understand. This would require a detailed knowledge of the lipid composition of those membranes which are involved in the rapid growth of pollen tubes. If the decrease of phospholipid quantity is limited to hydrated, but ungerminated grains, as discussed above, then this aspect would have no relevance for radioactive experiments which reflect activities of living cells only.

Despite these uncertainties we summarize and interprete our data in the following way. During hydration of pollen grains, the quantity of membrane lipids is increased to some extent without a concomitant increase in membrane area. The subsequent initiation of tube growth enlarges the surface of the plasmamembrane by factors of five to 10 within the four hr of our experiment. This increase in membrane area is not paralleled by a rise in the quantity of membrane lipids or an increase in labelling rate. This would indicate that, apart from some *de novo* made, mainly pre-existing membrane lipids are used for the extension of the plasmamembrane. This explanation is supported by the suggestion that the numerous, electron-dense and spherosome-like deposits

in pollen grains contain lipid precursors for synthesis of membrane lipids following pollen germination [22]. In addition, the intravacuolar concentric membrane bodies and the large quantities of closely stacked rough endoplasmic reticulum, both observed in pollen grains and very young tubes, may serve as stores of membrane material for rapid activation [23, 24] during hydration and tube growth initiation. This may also explain the very low content of glycolipids such as SG, ASG and CE which account for 10–35% of lipids from plasmamembranes surrounding parenchymatous cells [25–27].

EXPERIMENTAL

Plant material. Pollen of *Nicotiana sylvestris* (Speg. and Comes var. SCR) was collected during early stages of anthesis from plants grown outdoors in the Botanical Garden of Hamburg. Dry pollen (*ca.* 100 mg) was suspended in an aq medium (20 ml, 0,3 M sucrose, 3 mM $Ca(NO_3)_2$ and 0.01% H_3BO_3) for germination and tube growth at 25° in the dark.

Lipid extraction and separation. The aq medium containing pollen grains was boiled for 10 min and after cooling sonicated for 5 min. The resulting suspension was used for lipid extraction [8]. The organic phase was evapd to dryness and is referred to as total lipid extract (TL). It was redissolved in $CHCl_3$–MeOH (2:1; 1 ml) and sepd into lipid classes by CC on silicic acid (Merck Kieselgel 60). The column (0.5 × 8 cm) was sequentially eluted with $CHCl_3$ (15 ml) to obtain the neutral lipid fraction (NL), with Me_2CO (20 ml) to yield the glycolipid fraction (GL) and finally with MeOH (20 ml) to obtain the phospholipid fraction (PL). Each fraction was analysed by TLC using petrol–Et_2O–HOAc (40:10:1) for NL; $CHCl_3$–MeOH (4:1) first dimension and C_6H_6–Me_2CO–H_2O (65:25:1) second dimension for GL and $CHCl_3$–MeOH–HOAc (65:25:8) for PL. Spots were visualized with anilinonaphthalene sulphonate (0.2% in MeOH) under UV light. Identification of individual lipids was achieved using different spray reagents [28] and by co-chromatography with standards available commercially or from previous investigations [8].

Quantitative analyses. Glycolipids were quantified by a modified anthrone method [29]. After 2D-TLC, individual glycolipid spots were scraped off into screw-cap tubes. Anthrone reagent (2 ml; 100 mg anthrone in 50 ml conc. H_2SO_4 and 25 ml H_2O) was added and the tubes heated for 20 min at 85°. After cooling and short centrifugation the *A* of the supernatant fluid was measured at 625 nm. Glucose and galactose served as standards. Phospholipids were quantified colorimetrically according to ref. [30] using disodium phenyl phosphate as std. Fatty acid Me esters, obtained by acid methanolysis of individual lipids, were sepd by GC as described before on Reoplex 400 as stationary phase [8].

Labelling experiments. For incorporation of radioactive precursors, 3 μCi of [1-^{14}C] acetate (sp. act. 57 μCi/μMol) or [^{32}P] phosphoric acid (sp. act. 200 μCi/μMol) were added per ml of germination medium. TLC chromatograms were scanned for radioactivity and radioactive spots scraped off into scintillation fluid (Quickszint 701, Zinsser, F.R.G.) for scintillation counting.

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