Transient increase of phosphatidylcholine in plant cells in response to phosphate deprivation

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Abstract In plants, phosphate deprivation is normally known to decrease the phospholipid content consistent with a mobilization of the phosphate reserve, and conversely to increase non-phosphorous membrane lipids such as digalactosyldiacylglycerol. We report here that unexpectedly, at an early stage of phosphate starvation, phosphatidylcholine (PC) increases transiently. We also show that a significant pool of diacylglycerol (DAG) with the same fatty acid composition as that of PC is present and moreover increases in response to phosphate deprivation. The evolution of the molecular profile of the newly synthesized galactolipids is compatible with a utilization of DAG accumulating from PC hydrolysis, achieved after selection of their acyl molecular species by the galactolipid synthesizing enzymes.

Key words: Plant cell; Phosphate deprivation; Membrane; Galactolipid; Phosphatidylcholine; Chloroplast

1. Introduction

Phosphorus is a basic element for plant growth and development. In most soils, in spite of its abundance, phosphorus is not freely available for assimilation by roots [1,2], and is often a limiting factor for land plants. In response to phosphate deprivation, plant cells activate integrated systems that facilitate the overall uptake of free inorganic phosphate (Pi) and reduce the metabolic phosphate consumption. Effects on membrane composition and dynamics have been described such as: (a) an overall decrease in phospholipid content consistent with a mobilization of the Pi reserve in these molecules [3], and conversely (b) an increase in some non-phosphorous lipids commonly acknowledged as unique to plastids, such as digalactosyldiacylglycerol (DGDG) [4,5]. The DGDG increase is not simply due to its relative balance after the phospholipid decrease. Indeed, several genes necessary for the syntheses of DGDG and its precursor, i.e. monogalactosyldiacylglycerol (MGDG), were shown to be activated by Pi deprivation [6,7], indicating the existence of a −Pi-induced pathway for the synthesis of plastid galactolipids.

Plastid galactolipids consist of two main molecular species characterized by a hydrophobic 'signature' at the *sn*-2 position of the glycerol backbone: the major one has 18-carbon (C_{18}) fatty acids (FAs) esterified at the *sn*-2 position of the glycerol backbone, the second a C_{16} FA at the *sn*-2 position. Relative to the *sn*-1/*sn*-2 FAs from the hydrophobic moiety, the C_{16}/C_{18} or C_{18}/C_{18} structure is found in all eukaryotic lipids. By contrast, the C_{16}/C_{16} or C_{18}/C_{16} structure is similar to that of cyanobacterial glycerolipid and is therefore called prokaryotic. Plants such as *Acer pseudoplatanus*, containing only eukaryotic galactolipids are called 'C18:3 plants' whereas others, such as *Arabidopsis thaliana*, contain also prokaryotic galactolipids and are referred to as 'C16:3 plants'. Two distinct pathways lead to the prokaryotic and to the eukaryotic basic structures of 1,2-*sn*-diacylglycerol (DAG), and as a consequence, to the galactolipids directly produced from DAG owing to the MGDG synthase and DGDG synthase activities. The envelope membranes which surround plastids contain all the enzymatic activities responsible for the biosynthesis of prokaryotic DAG, whereas it is commonly assumed that the eukaryotic DAG available for plastid galactolipid biosynthesis originates from an extraplastidic pool, generated from phosphatidylcholine (PC) [8,9]. In this respect, the FA composition of DGDG produced after Pi deprivation was shown to be enriched in C_{18} at the *sn*-2 position and C_{16} at the *sn*-1 position of the glycerol backbone [10,11], a signature that suggests a synthesis from a DAG hypothetically originated from non-plastidial PC. In addition, although DGDG is considered as unique to plastid membranes, the newly synthesized DGDG was proposed to replace missing PC in cell membranes after relocation outside plastids [11]. A very recent study shows that, upon Pi deprivation, oat membrane fractions enriched in plasma membranes accumulate tremendous amounts of DGDG, up to 70% of the total plasma membrane glycerolipid content [12]. This report adds to the list of arguments in favor of a net synthesis – and possibly transfer – of DGDG dedicated to extraplastidic membranes when phosphate is limited.

Many data are missing to assess the precise route of the hydrophobic moieties of phospholipids and galactolipids that are reshaped in response to Pi deprivation, at the level of their phosphorus-containing and phosphorus-lacking polar heads. In this study, we analyzed the evolution of each lipid class, and their relative FA content, after transfer of a C18:3 or a C16:3 plant cell culture into Pi-deprived medium. We report the unforeseen increase of PC in the first stage after transfer

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Abbreviations: FA, fatty acid; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; DAG, diacylglycerol; PE, phosphatidylethanolamine; DPG, cardiolipin; PG, phosphatidylglycerol; PI, phosphatidylinositol; FAME, fatty acid methylated ester; Pi, inorganic phosphate; Cells grown without Pi are qualified as '−Pi' whereas those grown with 4 mM (for *Acer pseudoplatanus*) and 1 mM (for *Arabidopsis thaliana*) of Pi are considered as '+Pi' controls

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into Pi-deprived medium. Focusing on the FA signatures, the transient PC increase correlates with the concomitant production of DAG of similar molecular profile. During Pi deprivation, the evolution of the molecular profile of the newly synthesized galactolipids is compatible with a utilization of DAG accumulating from PC hydrolysis, and achieved by the galactolipid synthesizing enzymes, after a selection of the acyl molecular species strictly monitored at the level of the galactolipid synthesizing enzymes.

2. Materials and methods

2.1. Plant material

An *A. pseudoplatanus* cambium cell suspension [13] was cultured in media defined by [14] and modified by [13]. Cells were maintained as 250-ml cultures on a rotational shaker (125 rpm). Cells were washed three times with 200 ml of fresh media containing either 0 or 4 mM Pi and subsequently transferred into fresh media containing either 0 or 4 mM Pi. A photosynthetic *A. thaliana* cell suspension [15] was cultured in media defined by Gamborg [16]. Cells were maintained as 250-ml cultures on a rotational shaker (125 rpm) with permanent light. Cells were washed three times with 200 ml of fresh media containing either 0 or 1 mM Pi and cultured in fresh media containing either 0 or 1 mM Pi. *A. pseudoplatanus* was used as a model for a 'C18:3 plant' and *A. thaliana* for a 'C16:3 plant'.

2.2. Lipid analysis

Glycerolipids were extracted from 2 g of harvested cells, according to [17]. Lipids were then separated onto glass-backed silica gel plates (Merck) by two-dimensional chromatography. The first migration was with chloroform:methanol:water (65:25:4, v/v), and the second with chloroform:acetone:methanol:acetic acid:water (50:20:10:10:5, v/v). Lipids were visualized under ultraviolet (UV) light after pulverization of 8-anilino-1-naphthalenesulfonic acid, 2% in methanol, and identified by comparison with standards. To separate DAG, lipids were run on silica gel plates (Merck) with di-isopropylether:acetic acid (96:4, v/v).

2.3. FA quantification

Known amounts of C21:0 were added to lipids scraped off the plate and FAs were methylated using 3 ml of 2.5% H_2SO_4 in methanol during 1 h at 100°C. Reaction was stopped by addition of 3 ml of water and 3 ml of hexane. The hexane phase was analyzed by gas liquid chromatography (Perkin Elmer) on a BPX70 (SGE) column. Fatty acid methylated esters (FAMEs) retention times were compared with those of standards.

2.4. ^{31}P-nuclear magnetic resonance (NMR) spectroscopy

Dried lipid extracts from 2 g of cells were dissolved in 2475 μl chloroform, 450 μl methanol and 75 μl deuterated water. ^{31}P-NMR spectra were obtained at 162 MHz on a Bruker AMX400 spectrometer (Bruker, Billerica, MA, USA) at 20°C. The deuterium resonance of ^2H_2O was used as a lock signal. Acquisition parameters were: 70° pulse, 15 μs, repetition time 3.6 s, spectral width 8200 Hz, number of scans 256 (15 min), Waltz-16 ^{1}H decoupling sequence. Free induction decays were collected as 8K data points, zero filled to 16K, and processed with a 0.2-Hz exponential line broadening. A 20-s recycling time was used to obtain fully relaxed spectra and calibration of the peak intensities was performed by the addition of known amounts of the authentic compounds.

3. Results

In this study, we took advantage of cell suspension models that allow a fine control of Pi feeding. When *A. pseudoplatanus* cells are transferred to a Pi-free medium, the growth is maintained for about 2 days, corresponding roughly to a single cell division, and becomes progressively slower than that of +Pi control cells. After 2 days, −Pi cell growth is completely stopped. In contrast, +Pi control cells undergo such a rapid exponential phase of growth that a dilution by culture medium is made necessary after 7 days of culture. The amounts of total FAs, and of C18 and C16 FAs, parallel the growth curve in each condition, leading to stabilization in the amounts of C18 and C16 FAs after 2 days of culture under Pi deprivation (Fig. 1). These observations are consistent with the known property of Pi as being a limiting factor for plant cell division [18]. Fig. 2A shows the evolution of polar lipid distribution in *A. pseudoplatanus* in −Pi and +Pi conditions over 11 days of culture. Control cells show a high stability in the lipid class distribution, including phospholipids, glycolipids and DAG. By contrast, the lipid class distribution in −Pi cells is quickly modified. On the one hand, a complex redistribution of phospholipid classes is noticed. The phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) proportions decline early, after 1 day of starvation, and keep on declining until the end of the experiment. Phosphatidylinositol (PI) undergoes a slight decrease during the first day, then restores to its initial value after 3 days. The cardiolipin (DPG) proportion remains stable, as if this mitochondrial phospholipid was unaffected by the Pi deprivation. Most surprisingly, the PC proportion increases significantly during the first day of starvation and remains high for 3 days, before decreasing below its initial level. ^{31}P-NMR analysis confirmed the metabolic dynamics of phospholipids, i.e. the early decrease in PE and PG and the unexpected transient increase of PC following Pi deprivation (Fig. 3). On the other hand, in the case of phosphate-free glycolipids, the sulfolipid and galactolipid proportions raise significantly after

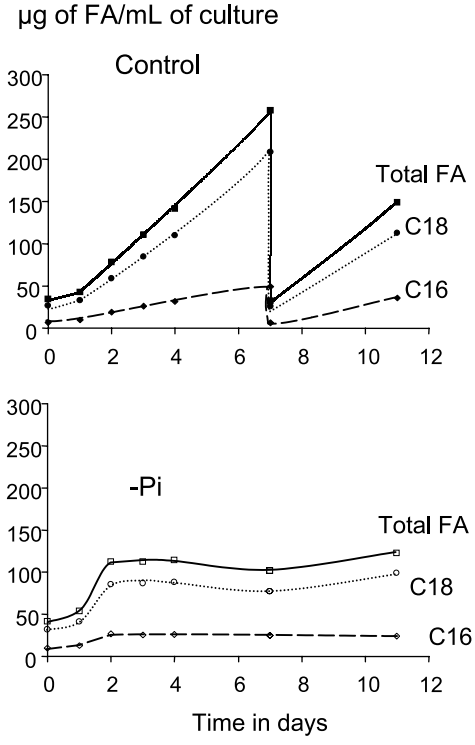

Fig. 1. Time course evolution of FA amount in *A. pseudoplatanus* cell culture. Cells were grown for 11 days from a concentration of 17 mg fresh weight/ml in media containing either 0 or 4 mM Pi. After 7 days of culture, they were transferred to a fresh identical medium without dilution for the −Pi cells and with an eight-fold dilution for +Pi cells.

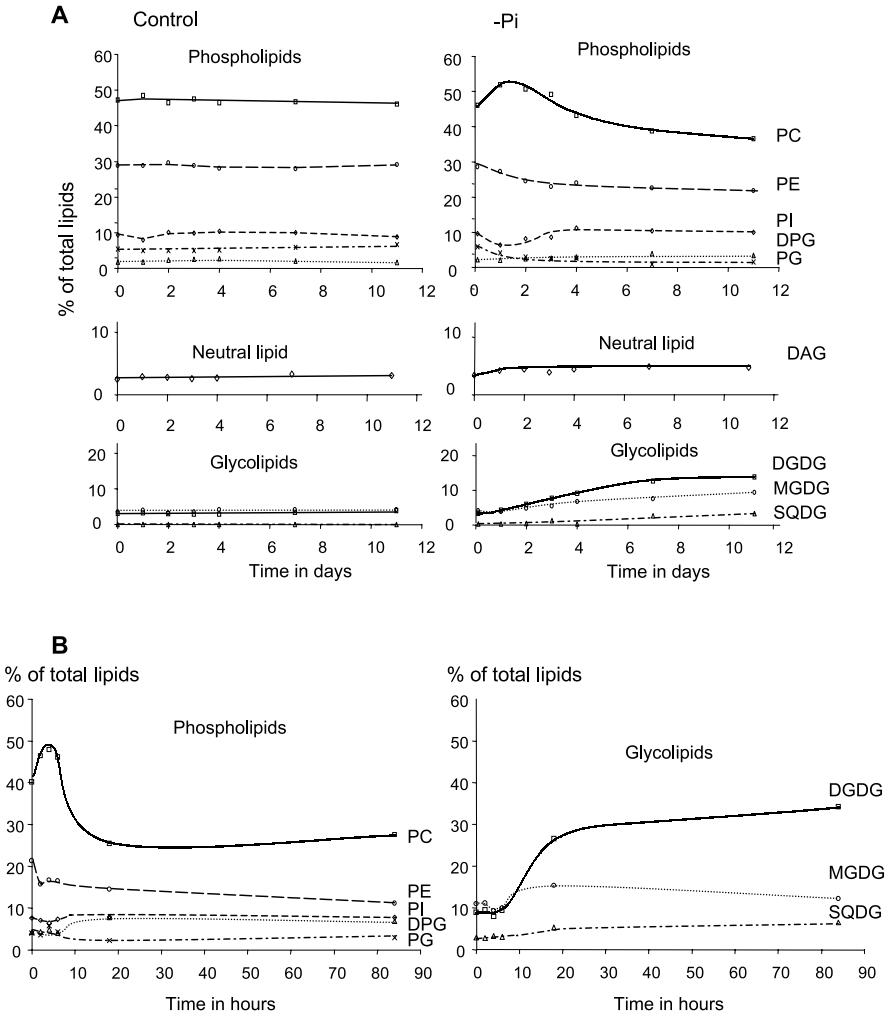

Fig. 2. Evolution of lipid content of plant cells grown as a suspension in media with or without Pi. Phospholipids, glycolipids and DAG were analyzed by thin layer chromatography (TLC) and FAME gas chromatography. A: Evolution of lipid composition in *A. pseudoplatanus* cell culture grown as described in Fig. 1. Results for days 0, 1 and 2 are average measurements of respectively four, three and two experiments with a maximum deviation of 5% for PC and PE, 10% for DPG, PG, MGDG and DGDG and 15% for PI. B: Three different batches of *A. thaliana* cells previously cultivated in a medium containing 1 mM Pi were grown respectively for 2–4–6 h, 18 h and 4 days from a concentration of 10 mg fresh weight/ml in a medium depleted of Pi. Since some variations were observed in the initial composition of cells, lipid composition of −Pi cells was reported to an initial composition average.

the first day of culture (Fig. 2A). This increase is particularly marked for DGDG.

PC is considered as the most potent source of DAG for plastid galactolipid synthesis through the eukaryotic pathway [8]. Therefore a C18:3 plant, such as *A. pseudoplatanus*, should synthesize galactolipids through this pathway. In support to this hypothesis, we noticed that the overall content of DAG raises from 2.5 to 5% of total lipids (Fig. 2A).

A. thaliana is a C16:3 plant and therefore can synthesize galactolipids, not only through the eukaryotic pathway, but also through the chloroplastic prokaryotic pathway that does not involve PC. In addition, *A. thaliana* are green cells and contain a higher proportion of plastid membranes in their chloroplasts, compared to sycamore cells that contain only amyloplasts. A higher proportion of galactolipids is therefore detected in control conditions. When following the lipid class composition of *A. thaliana* cells grown in presence or absence of phosphate, we observed that, in −Pi conditions, the PC transient increase does exist. Moreover the shift between the initial PC metabolic steady state and the −Pi-induced PC fall down occurs much faster than in *A. pseudoplatanus* (Fig. 2B). The PC increase peak is measured as early as 2 h after transfer in the −Pi medium. Maximum PC concentration is concomitant with the beginning of DGDG increase, 4 h after transfer. In addition, we noticed much more intense DGDG increase and PC decrease in the Arabidopsis cells than in the sycamore cells. Together, the experiments on the C16:3 and the C18:3 plants indicate that the −Pi-induced PC transient increase precedes closely the increase of galactolipids, independently of the occurrence of a prokaryotic pathway for galactolipid synthesis. PC is likely the most important glycerolipid that is altered under Pi deprivation to generate the DAG potentially used as a source for galactolipid synthesis. In agreement with this scenario, and similarly to the *A. pseudoplatanus* response, DAG increased in Pi starved *A. thaliana* cells (5.5% of total lipids after 2.5 days of deprivation versus 2.7% in control).

To further investigate the potent role of PC as a donor of the hydrophobic moiety of galactolipids induced by Pi deprivation, we analyzed the FA composition of polar lipids after phosphate removal in sycamore cell medium (Fig. 4A). In

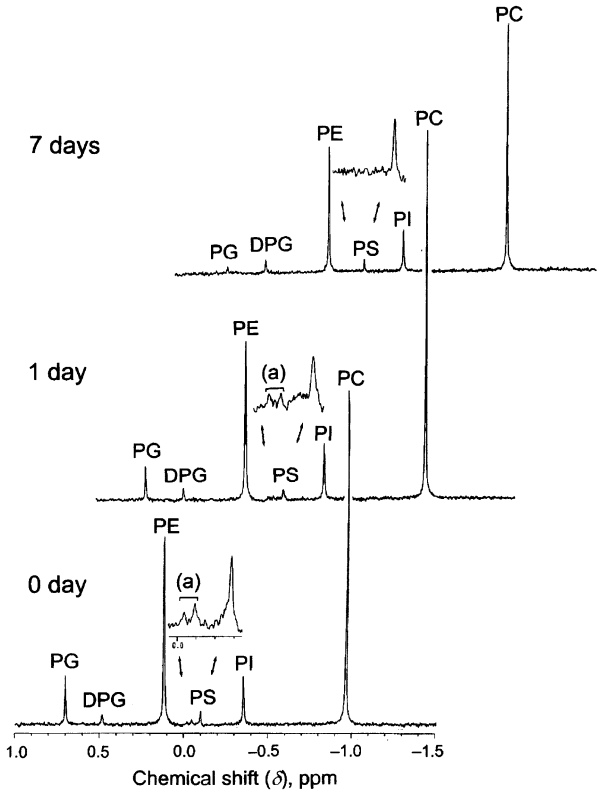

Fig. 3. Representative in vitro ^{31}P-NMR spectra of phospholipids of *A. pseudoplatanus* cells grown for indicated times in −Pi medium. Peak assignments were made from a series of spectra obtained after the addition of authentic compounds, using different proportions of methanol/H_2O in the solvent. (a): this pair of peaks corresponds to mono-methyl PE and di-methyl PE.

initial conditions (0) phospholipids such as PE, PC, and PG contain substantial proportions of C16:0, an acyl species which (a) is always esterified at the *sn*-1 position of glycerol and (b) is not desaturated in C18:3 plants. Conversely, the initial content of C16:0 in galactolipids is far lower (Fig. 4A). We considered therefore the peculiar enrichment in C16:0 as an initial signature of an extraplastidial origin for hydrophobic moiety transfers (Fig. 4B). Although the proportion of C18:2 is also higher in phospholipids, the C18:2 could not be used as an unambiguous signature since it could be transferred and/or desaturated into C18:3. Indeed, an overall decrease of C18:2 and increase of C18:3 were observed in total lipids of −Pi cells (not shown) suggesting stimulation in this desaturation step by Pi removal. After 2 days of deprivation, the C16:0 proportion increased in DGDG. In MGDG, the bulk FA composition was rather stable, although we detect a modest increase in C16:0, very early after Pi deprivation (1–2 days). By contrast, a decrease in C16:0 proportion occurred in PE and PG concomitantly with a bulk decrease of the respective phospholipid classes. PC, which underwent an accumulation peak, also exhibited a transient increase in C16:0. These results suggest that the loss of C16:0-containing molecular species from PE and PG is led by an early conversion into PC, where C16:0 accumulates momentarily, followed by a transfer to plastid galactolipids and particularly to DGDG in which C16:0 is loaded eventually. The acyl distribution in DAG is strikingly similar to that of PC (Fig. 4A) suggesting that it derives directly from PC, and that it is conceivably the source for the newly synthesized galactolipids, after a rigorous acyl molecular species selection at the level of MGDG and DGDG syntheses.

4. Discussion

When a land plant suffers from a sudden lack in phosphate, the root surface is exposed to the phosphate shortage before innermost and remote tissues. The earliest metabolic changes that occur in response to Pi deprivation are therefore far more complex at the level of the whole plant and therefore difficult to analyze. To circumvent this technical problem, the present study was carried out using plant cell suspensions and provides a comprehensive analysis of the fate of the polar head and hydrophobic moieties of major glycerolipid classes under Pi deprivation.

In plant cell cultures deprived of phosphate, the late glycerolipid evolution shows similar features as that documented at the level of whole plants, i.e. a decrease in phospholipids, particularly PC and an increase in galactolipids, particularly DGDG [4,5]. The release of the polar heads from phospholipids is supposed to feed the cell with phosphate. Concerning the early events, the most striking response is found at the level of PC, the main phospholipid present in all types of membranes except from plastid and mitochondria inner membranes, which undergoes a substantial transient increase after phosphate removal (Fig. 2). This early PC peak was observed in both *A. pseudoplatanus* and *A. thaliana*, C18:3 and C16:3 plants respectively. In contrast, major phospholipids like PE and PG are rapidly and highly reduced consistent with a mobilization of the phosphate reserve. DPG, a phospholipid unique to mitochondria, keeps rather constant, illustrating the relative stability of mitochondria and of respiration already noticed during Pi deprivation [3].

The transient increase of PC measured after Pi deprivation appears as a transitory state between an initial steady state of synthesis consumption and a new metabolic state. During the early transition, the PC accumulation involves a stimulation of the PC production and/or an inhibition of the PC utilization. After the accumulation peak, PC is rerouted to a new metabolic pathway in which its consumption is faster than its production: the simplest hypothesis is that the new metabolic utilization of PC is a massive hydrolysis to generate DAG that shall be a source for the hydrophobic moiety of −Pi-induced galactolipids.

In *A. pseudoplatanus* (Fig. 4A) and *A. thaliana* (not shown), we observed that DGDG produced during Pi deprivation is enriched in C16.0 and C18:2 FAs. In C18:3 plants such as *A. pseudoplatanus*, C16:0 is esterified at the *sn*-1 position of the glycerol backbone [19]. Likewise, in *A. thaliana*, Härtel et al. [5] demonstrated that C16:0 was also positioned at the *sn*-1 position. According to the acyl profiles, and particularly the C16:0 signature (Fig. 4B), the decrease of PE was selective: the net content of C16:0 fell clearly in PE. Evolution of FA in PG was relatively more complex but considering the net total decrease of PG, net content of C16:0 fell also in PG. On the opposite, C16:0 seemed transitorily stored in PC. The simplest scheme to explain the early accumulation into PC is therefore a direct or indirect conversion of other phospholipids into PC prior to a hydrolysis into DAG. For instance, PE can be methylated or converted into PC by a polar head exchange. Interestingly, we observed that mono-methyl and di-methyl

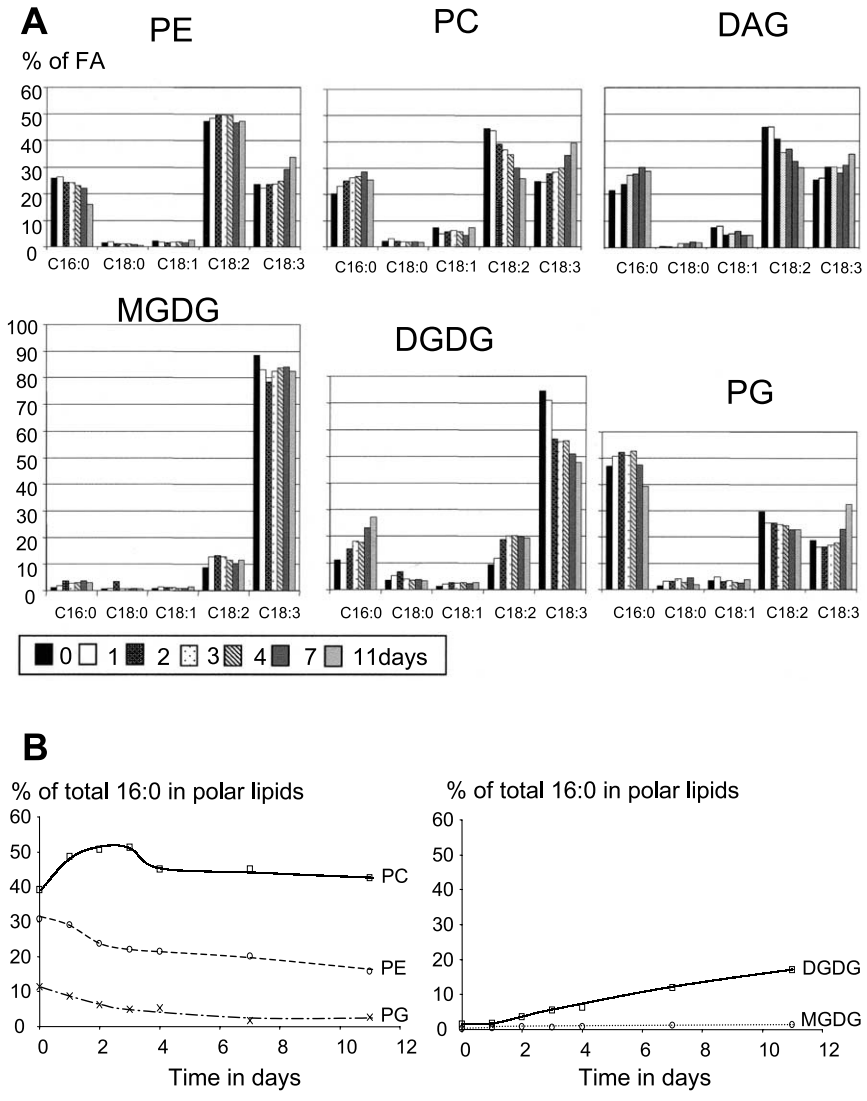

Fig. 4. Time course evolution of FA composition of *A. pseudoplatanus* cells submitted to Pi deprivation. A: Evolution of FA composition of single polar lipids MGDG, DGDG, PC, PE, PG and DAG. Results are expressed in % of total FAs in a single lipid. B: Evolution of C16:0 distribution among polar lipids. Results are expressed in % of total C16:0 in polar lipids. In control cells, C16:0 distribution remained stable during the culture.

PE visualized by ^{31}P-NMR analysis in the control declined after the first day of Pi deprivation, and were no more apparent after 7 days of Pi deprivation (Fig. 3), suggesting that PE methyltransferase activity is stimulated.

The enrichment of galactolipids in eukaryotic structures is particularly marked in DGDG. Surprisingly, MGDG does not undergo any in-depth redistribution of its acyl chains besides a small increase in C16:0. We speculate therefore that the −Pi-induced synthesis of DGDG is channeled, owing to a very dynamic and straightforward transitory synthesis DAG → MGDG → DGDG. Meanwhile, the initial MGDG pool does not seem mobilized during the Pi deprivation time course: our results suggest therefore that an independent pool of MGDG is transitorily produced and directly consumed for the production of the −Pi-induced pool of DGDG.

DGDG synthesis correlates with −Pi-stimulated expression of genes involved in galactolipid synthesis, i.e. MGDG synthases of type B (*mgd2* and *mgd3*) and DGDG synthases (*dgd1* and *dgd2*) [6,7]. The observation that AtMGD2 and AtMGD3 have a higher specificity for eukaryotic than for prokaryotic DAG molecular species [6] supports that these enzymes can select DAG molecules and form MGDG necessary for the production of the −Pi-induced DGDG. It was previously suggested that AtMGD2 and AtMGD3 could be associated with the outer membrane of the plastid envelope and that AtDGD1 and AtDGD2, involved in the conversion of MGDG to DGDG, are located in this same membrane [20,21]. This probable colocalization, together with the lipid analysis documented in this paper, gives a major role to the plastid outer membrane in the production of DGDG during Pi deprivation. A transfer of DGDG to extraplastidic membranes, such as the plasma membrane [12], would therefore recruit a lipid transfer machinery that remains to be characterized.

This work gives a new focus on the recurrent and unanswered question of the source of eukaryotic glycerolipid structures in plastids. Although PC is present in most cell membranes besides inner membranes from plastids and mitochondria, it is uniquely synthesized in the ER [22]. Two mechanisms were previously proposed to explain a potent

transfer of DAG generated from PC to plastid galactolipids, either a transport of PC from ER to plastid envelope membranes [23] or a transport of Lyso PC [24,25]. To date, the molecular aspects of these transport mechanisms remain obscure. The question is made even more complex due to the abundance of PC phospholipase A2 and Lyso PC acyltransferase in most cell membranes that lead to an important FA exchange at the *sn*-2 position of PC. Indeed, Williams et al. [9] emphasized the importance of FA exchange between PC and acyl CoA in the composition of DAG ultimately incorporated into plastid galactolipids. In our experiment, we noticed that (a) a pool of DAG is generated with the same FA composition as that of PC, and (b) this pool increases in response to Pi deprivation. We conclude therefore that (a) DAG is seemingly in equilibrium with PC without any specificity regarding the hydrophobic moiety, and (b) that the equilibrium is displaced towards DAG during Pi deprivation. It would be interesting to determine the exact localization of this pool of DAG. One favored hypothesis is that DAG is located outside the plastids since DAG was not substantially detected in isolated intact chloroplasts [26] or non-green plastids [27]. This would therefore indicate that a direct transfer of DAG occurs from non-plastid membranes towards the plastid envelope and that this transfer step is limiting.

All together this work enlightens the role of PC in plant galactolipid synthesis and shows for the first time that PC plays a function in the early response of the plant to Pi deprivation. The fine analysis of phosphate regulation of enzymes manipulating PC, like PE methyltransferase or PC phospholipases *c* or *d*, will certainly help to understand the early events of the plant cell adaptation to Pi deprivation.

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