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Long- and short-term phosphate deprivation in bean roots: Plasma membrane lipid alterations and transient stimulation of phospholipases

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Abstract

In a long-term experiment bean (*Phaseolus vulgaris* L.) seedlings were grown for 18 days in hydroponics in either phosphate-sufficient (+P) or phosphate-deficient (−P) nutrient solutions. Phosphate deprivation halved the phosphorous content of roots. In plasma membrane (PM) fractions isolated from −P roots the phospholipid (PL) level was reduced from 35 to 21 mol%, while PL composition and degree of unsaturation were hardly altered. Digalactosyldiacylglycerol (DGDG) accumulated up to 26% of total PM lipids, replacing PL to a large extent. Molecular species and fatty acid compositions of DGDG in root PM were different compared to DGDG present in the −P plastids. In a short-term study, bean seedlings were grown for 18 days in hydroponics with a complete nutrient solution containing phosphate and then incubated in a −P medium for increasing time ranging from 1 up to 96 h. At the end of the starvation period phosphorous content of −P roots was reduced by 30% compared to +P ones. An activation of phospholipase D and phospholipase C was observed after 1 and 2 h of phosphate deprivation, respectively. Maximal phosphatidic acid accumulation was detected after 4 h of phosphate deprivation, when also DGDG started to accumulate in PM of bean roots. The fatty acid composition of PLD-derived phosphatidylbutanol resembled that of phosphatidylcholine.

Keywords: *Phaseolus vulgaris*; Leguminosae; Bean; Digalactosyldiacylglycerol; Phosphate deprivation; Phospholipase C; Phospholipase D; Phospholipids; Plasma membrane; Roots

1. Introduction

Phosphorous is an essential nutrient for plant growth. In spite of its abundance, most of it is immobile in the soil and not freely available for root uptake (Martin et al., 2000). Since phosphate (P_i) is a component of key molecules such as nucleic acids, phospholipids (PL) and ATP, plants cannot grow without a reliable supply of this metabolite. Plants have evolved integrated adaptive responses to P_i limitation that are active at both morphological and metabolic levels. Morphological adaptations are represented by an increase in the root to shoot ratio, which is the result of shoot growth reduction and root growth stimulation, and changes in root architecture (Nakamura et al., 2005). Metabolic responses to P_i limitation are directed to maintain constant cytoplasmic P_i concentration, to enhance P_i uptake as well as synthesis and secretion of root exudates and to activate enzymes that increase phosphorous availability and P_i remobilization (Malusà et al., 2006).

Lipids are integral components of membranes and are fundamental for their structural and functional integrity. A decrease in the PL content of membranes is a common response of plants to phosphorous deprivation aimed to mobilise the P_i reserve from these molecules (Andersson

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et al., 2005; Cruz-Ramirez et al., 2006; Li et al., 2006). It has been established that when P_i is limiting, the decrease in PL is compensated for by an increase in the non-phosphorous digalactosyldiacylglycerol (DGDG), usually acknowledged as unique to plastids (Härtel et al., 2000, 2001). This change in lipid composition is not restricted to plastids, because during P_i deprivation DGDG was found to accumulate in extraplastidial membranes such as plasma membrane (PM), tonoplast and mitochondria (Jouhet et al., 2003, 2006; Andersson et al., 2003, 2005; Kobayashi et al., 2006). These results suggest that the glycolipid DGDG replaces PL under P_i deprivation, and indicate the importance of DGDG in extraplastidial membrane systems in order to sustain P_i-requiring cellular processes. It has been hypothesised that the large amount of DGDG that accumulates in PM during P_i deprivation is derived from a DGD2 synthase activity localised at the chloroplast envelope membrane level, and that DGDG might be transported from the chloroplast to extraplastidial membranes (Kelly et al., 2003; Kelly and Dörmann, 2004).

Under altered environmental conditions, PL hydrolysis, besides representing a negative event in cell activity, is also involved in many vital cellular processes and plays a fundamental role in the formation of signal messengers (Quartacci et al., 2002; Wang, 2002; Navari-Izzo et al., 2006). Phospholipase D (PLD, EC 3.1.4.4) hydrolyses PL at the terminal phosphoesteric bond, generating phosphatidic acid (PA) and free head groups such as choline, whereas phospholipase C (PLC, EC 3.1.4.11) hydrolyses the PL phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerols (DAG) (Testerink and Munnik, 2005). These latter may represent moieties for galactolipid synthesis and/or may be rapidly phosphorylated in the membranes to PA by a DAG kinase. It is now clear that one of the roles of phospholipases is to provide an intracellular signal for cell activation represented by PA (Sang et al., 2001; Testerink and Munnik, 2005; Navari-Izzo et al., 2006). Thus, lipid messengers, when produced immediately after the stress and only for a short time, play positive effects in plant mediating and/or activating defence responses (Sang et al., 2001).

The aim of this study was to monitor alterations of bean root PM lipids following both long and short periods of P_i deprivation in order to establish the role of DGDG in the replacement of PL in extraplastidial membranes. In addition, we evaluated changes in root PLC and PLD activities to verify whether they may represent an early event in the lowered P_i status signalling events.

2. Results

2.1. Long-term phosphate deprivation

In bean seedlings grown for 18 days in hydroponics in either P_i-sufficient or P_i-deficient nutrient solutions the total phosphorous concentration of −P roots was 2-fold lower in comparison with +P roots (27 and 51 mg g^{-1} dry wt, respectively).

The main PM lipids of +P bean roots were PL and cerebrosides. In the −P roots PL decreased from 35.2 to 20.9 mol%, whereas cerebrosides (about 28 mol%) did not show any change in comparison with +P plants (Fig. 1a). Among steryl lipids, free sterols decreased from 18.9 to 11.3 mol%, while steryl glycosides and acylated steryl glycosides remained constant. DGDG, not detectable in the PM of +P roots, was present in the −P ones in the proportion of 26.0 mol% of total PM lipids (Fig. 1a). The predominant PL in root PM were phosphatydilcholine (PC) and phosphatydilethanolamine (PE). In spite of the appreciable decrease in PL content of root PM fractions after long-term P_i starvation, PL composition was altered only slightly. As depicted in Fig. 1b, the levels of PE and phosphatidylglycerol (PG) decreased while those of phosphatidylinositol (PI) and phosphatidylserine (PS) increased.

In the PM of +P roots the main fatty acids of PL were linoleic (18:2) and linolenic (18:3) acids, followed by lower

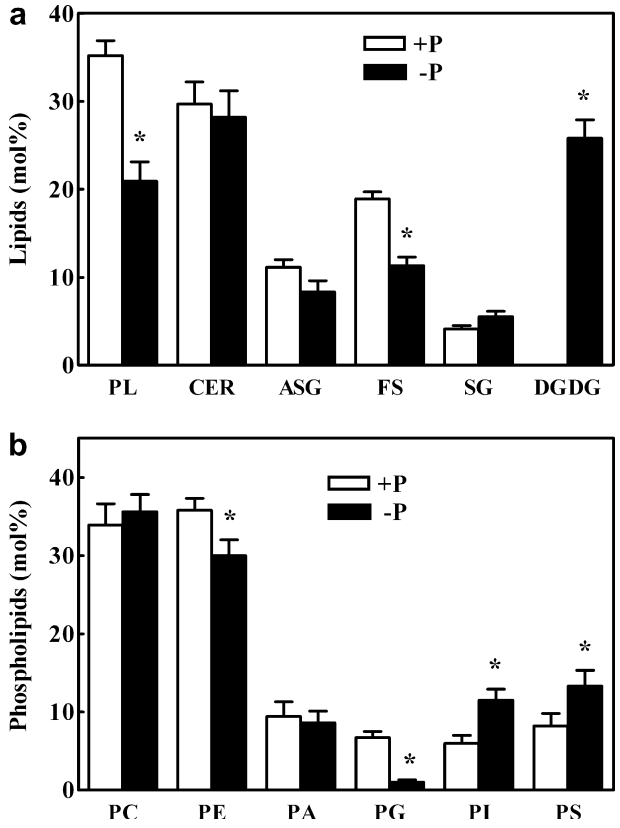

Fig. 1. Total lipid (a) and phospholipid (b) composition of plasma membrane isolated from bean roots. Seedlings were grown for 18 days on phosphate-sufficient (+P) or phosphate-deficient (−P) Knop solutions. Results are the means of three independent experiments each analysed twice ±s.d. ($n = 3$). Means followed by an asterisk are significantly different at $P \leqslant 0.05$. ASG, acylated steryl glycosides; CER, cerebrosides; DGDG, digalactosyldiacylglycerols; FS, free sterols; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; SG, steryl glycosides.

levels of palmitic (16:0), oleic (18:1) and stearic (18:0) acids (data not shown). After 18 days of P_i deprivation, 18:1 was drastically reduced from 16 to 3 mol%, while an increase in 18:2 (from 29 to 35 mol%) and 18:3 (from 27 to 34 mol%) was observed. Concerning PL unsaturation, P_i deprivation did not change the double bond index (DBI) compared to +P roots (5.6 and 6.0, respectively).

The fatty acid composition of DGDG consisted mainly of 16:0, 18:2 and 18:3 both in PM and plastids of −P roots (Table 1). Plastids showed higher amounts of 16:0 (48 mol%) and 18:3 (16 mol%) and lower amounts of 18:2 (32 mol%) in comparison with PM. In DGDG of root PM, 16:0 and 18:3 levels were lower (39 and 10 mol%, respectively), while an increase in 18:2 was observed. The main DGDG molecular species in plastids and PM of roots were 16:0/18:2, 18:2/18:2 and 18:2/18:3, while 16:0/18:3 and 18:3/18:3 were present in smaller proportions. Compared to plastids, DGDG of PM contained a higher proportion of 18:2/18:2 and a lower proportion of 16:0/18:2 species (Table 1).

2.2. Short-term phosphate deprivation

Bean seedlings were grown for 18 days in hydroponics with a complete nutrient solution and then incubated in a −P medium for increasing time (from 1 up to 96 h). Short-term P_i deprivation caused a decrease of total phosphorous concentration in the roots and after 96 h of P_i deprivation a reduction of about 30% (from 56 to 38 μg g^{-1} dry weight) was observed.

A very rapid accumulation of DAG was detected between 1 and 2 h of P_i deprivation (Fig. 2a). After 2 h DAG proportion was 4.6-fold higher compared to +P roots. Thereafter, DAG decreased to the value of control roots. DAG accumulation may be a consequence of the direct hydrolysis of PL by PLC or the result of a two-step reaction catalysed by PLD and PA phosphatase (PAP).

Table 1
Fatty acid composition (mol%) of digalactosyldiacylglycerol in plastids and plasma membrane isolated from bean roots

	Plastids	Plasma membrane
Fatty acid		
16:0	48 ± 1.7	39 ± 1.2*
18:0	2 ± 0.4	4 ± 0.6
18:1	2 ± 0.3	3 ± 0.3
18:2	32 ± 1.5	44 ± 2.0*
18:3	16 ± 1.1	10 ± 0.9*
Molecular species		
16:0/18:2	40 ± 1.7	33 ± 1.1*
16:0/18:3	9 ± 1.7	5 ± 1.5
18:2/18:2	16 ± 1.4	25 ± 1.8*
18:2/18:3	30 ± 1.9	34 ± 2.1
18:3/18:3	5 ± 1.2	3 ± 0.8

Seedlings were grown for 18 days on phosphate-deficient (−P) Knop solution. Results are the means of three independent experiments each analysed twice ±s.d. ($n = 3$). Means followed by an asterisk are significantly different at $P \leqslant 0.05$.

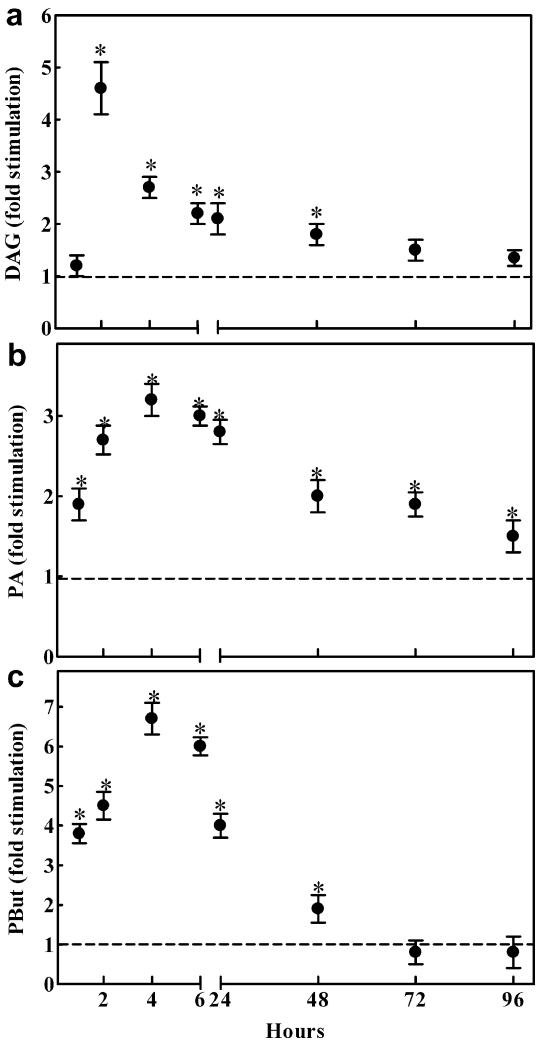

Fig. 2. Diacylglycerols (DAG) (a), phosphatidic acid (PA) (b) and phosphatidylbutanol (PBut) (c) in roots of bean. Seedlings were grown for 18 days on +P Knop solution and then incubated in −P solution for different periods of time (from 1 to 96 h). At each time point DAG, PA and PBut are expressed as fold stimulation in comparison with +P roots (horizontal line, 0.9 ± 0.1 mol% of total lipids for DAG, 9.4 ± 0.4 mol% of total PL for PA and 0.23 ± 0.02 mol% of total PL for PBut, respectively). Results are the means of three independent experiments each analysed twice ±s.d. ($n = 3$). Means followed by an asterisk are significantly different at $P \leqslant 0.05$.

Even if the activity of PAP did not change throughout the whole experiment (data not shown), DAG accumulation via PLD was still possible if PA represented a limiting factor in the two-step reaction. Moreover, it cannot be ruled out that DAG accumulation may also derive from an enhanced biosynthesis or from a reduced consumption. The PA level of roots increased already after 1 h of incubation in the −P solution, reaching the highest value of 30 mol% after 4 h (3.2-fold increase compared to +P roots), and decreasing thereafter (Fig. 2b). To analyse the role of PLD in the formation of PA, the enzyme activity was assayed (Fig. 2c). PLD has the unique ability to transfer the phosphatidyl group of its substrate, instead to water, to a primary alcohol forming phosphatidyl alcohols.

In the presence of 0.2% *n*-BuOH, the amount of phosphatidylbutanol (PBut), that is not normally found in biological membranes, is a relative measure of PLD. As for PA, also PBut accumulation increased during the first 4 h of P_i deprivation when it peaked to 6.7-fold in comparison with +P roots (1.5 mol% of total PL). PBut decreased to the initial value after 72 h of incubation (Fig. 2c).

After 4 h of P_i deprivation, when PLD showed its maximal activity, an increase in PM cerebrosides (from 30 to 44 mol% of total lipids) and acylated steryl glycosides (from 11 to 21 mol% of total lipids) occurred in roots. On the contrary, a significant decrease in PL and free sterols was observed (Fig. 3a). DGDG, not detectable in the +P roots, represented about 2 mol% of total lipids in the PM of −P roots. After 4 h of treatment, PC decreased from 38.9 to 28.8 mol%, while PE increased from 37 to 42.3 mol% (Fig. 3b). As a consequence, the PC to PE molar ratio decreased from 1.0 to 0.7. Following P_i deprivation, PI and PS remained constant, while PG decreased from 6.7 to 1.4 mol%. The proportion of PA increased reaching a value of about 18 mol% of total PL (Fig. 3b).

After 4 h of P_i deprivation, the fatty acid composition of PBut closely resembled that of PC, whereas PA and DAG showed higher proportions of 16:0 and 18:0 (Fig. 4).

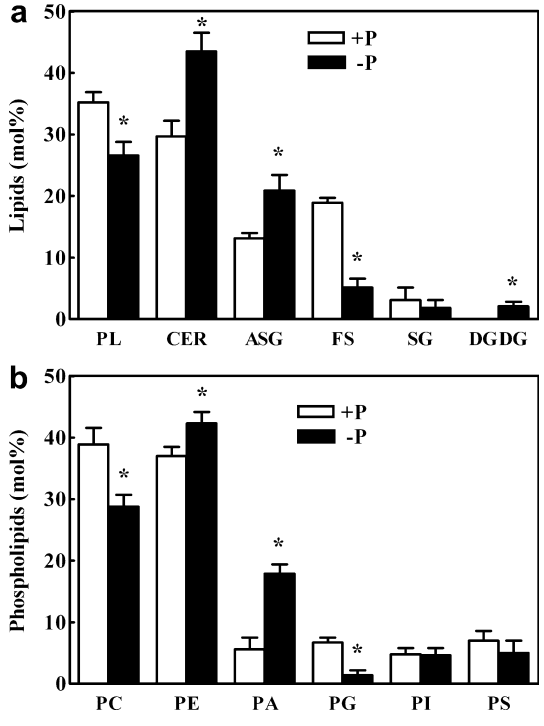

Fig. 3. Total lipid (a) and phospholipid (b) composition of plasma membrane isolated from bean roots. Seedlings were grown for 18 days on +P Knop solution and then incubated in −P solution for 4 h. Results are the means of three independent experiments each analysed twice ±s.d. ($n = 3$). Means followed by an asterisk are significantly different at $P \leqslant 0.05$. For abbreviations see Fig. 1.

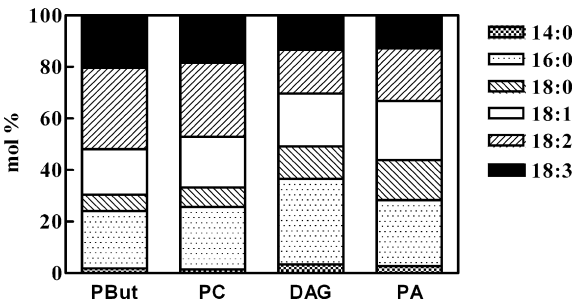

Fig. 4. Fatty acid composition of phosphatidylbutanol (PBut), phosphatidylcholine (PC), diacylglycerols (DAG) and phosphatidic acid (PA) of plasma membrane isolated from bean roots. Seedlings were grown for 18 days on +P Knop solution and then incubated in −P solution for 4 h. Results are the means of three independent experiments each analysed twice ±s.d. ($n = 3$). Means followed by an asterisk are significantly different at $P \leqslant 0.05$.

3. Discussion

3.1. Long-term phosphate deprivation

In our experiment the P_i amount after 18 days of P_i deprivation did not reach the very low concentration (6% of control plant) detected after 2 weeks in roots of a different bean cultivar (Gniazdowska et al., 1998). However, the two-fold reduction of phosphorous content had a negative effect on root growth being P_i a limiting factor for plant cell division (Sano et al., 1999). Indeed, −P roots were shorter and thicker, and showed an increased formation of side roots compared to +P plants (Malusà et al., 2006). These developmental responses, which increase the root surface area to volume ratio, result in a more profusely branched, shallow root system, and have been interpreted as an acceleration of spatial soil exploration (Abel et al., 2002).

In response to P_i deprivation, an overall decrease in PL proportion (Fig. 1a) may have mobilised the reserve of these compounds in order to feed the cells with P_i, and might have been a source of DAG for the synthesis of galactolipids replacing PM phospholipids (Nakamura et al., 2005; Cruz-Ramirez et al., 2006; Li et al., 2006). In *Arabidopsis thaliana* cell suspension PE was rapidly reduced by P_i deprivation with a simultaneous transient increase in PC, suggesting that PE could have been methylated or converted into PC by a polar head exchange (Jouhet et al., 2003). The subsequent hydrolysis of PC could have originated DAG and formed the hydrophobic moiety of DGDG (Andersson et al., 2003, 2005; Nakamura et al., 2005). In bean roots (Fig. 1b), phosphatidylglycerol was the PL more affected by P_i deficiency, similarly to what observed in *Arabidopsis* cell suspension and wheat leaves (Jouhet et al., 2003; Wang and Xu, 2006). The results of this study also suggest that cv. Bianco di Bagnasco is relatively tolerant to P_i deprivation as the alterations in PL composition (Figs. 1a and b) are less distinct than those observed in the PM of another bean cultivar grown for 19 days under P_i deprivation (Gniazdowska and Rychter, 2000). Interestingly, the

extent of PL-to-DGDG replacement (26 mol%) closely resembled that found in PM of oat roots after 4 weeks of P_i limitation (Andersson et al., 2005).

In our study DGDG was not detected in the PM of +P bean roots (Fig. 1a), differently from what observed in +P oat roots by Andersson et al. (2003) who found that DGDG represented 4 mol% of total PM lipids. It has been suggested that the presence of low levels of DGDG reflects a basal activity of DGDG synthesis for non-plastidial membranes. Even when +P conditions are aimed for, P_i may become a limiting nutrient, and lipid replacement thus initiates (Andersson et al., 2003). The different finding could be explained by the species investigated and the experimental procedures (membrane isolation, lipid separation) used. The DGDG from PM of −P roots had a fatty acid composition different from that of root plastids (Table 1), suggesting that the newly formed galactolipid was synthesised through the eukaryotic lipid pathway by MGDG and DGDG synthases localized in the outer plastid envelope and subsequently transferred to extraplastidial membranes (Härtel et al., 2000; Kelly and Dörmann, 2004; Cruz-Ramirez et al., 2006; Li et al., 2006).

The reduction of oleic acid observed in the total PL of −P roots (from 16.4 to 3.0 mol%) is probably the result of a re-distribution of double bonds among the different C_{18} fatty acids rather than a peroxidative process, being the total unsaturation of PM unaffected by the treatment.

3.2. Short-term phosphate deprivation

The increased PLD activity observed after one h of P_i deprivation (Fig. 2c) strengthen the hypothesis that PL, and PC in particular (Figs. 3a and b and 4), could have served as substrate for PA formation. However, the effects of PLD action may be different in response to the severity of P_i starvation (Li et al., 2006). Under severe conditions PC hydrolysis results in the formation of DAG for DGDG synthesis, whereas in moderate P_i deficiency PLD activity induces PA accumulation to stimulate root growth. Andersson et al. (2005) observed that a PLD-type with unusual properties and a PA phosphatase were the dominant lipase activities induced by P_i deficiency after 4 weeks of growth, and suggested that lipase activities were required to maintain the low PL content of the plasma membrane during prolonged P_i deprivation and were not part of a signalling cascade. In *A. thaliana* roots, a novel non-specific PLC, NPC4, was induced 6 days after plant transfer to a P_i-free medium (Nakamura et al., 2005). Also in this case, the broad substrate specificity and the fact that a major portion of DAG was not converted to PA indicates that NCP4 was mainly involved in membrane lipid degradation rather than signal transduction. In the same species, hydrolysis of PC by PLDζs during phosphorus deprivation contributed to the supply of DAG moieties for galactolipid synthesis in roots (Cruz-Ramirez et al., 2006; Li et al., 2006). The early enhancement of DGDG formation (Fig. 3a) agrees with the finding that in *A. thaliana* cell suspension DGDG accumulation started 4 h after transfer to the −P medium, suggesting that P_i limitation is quickly sensed by the root system at very low thresholds (Jouhet et al., 2003). In that study the beginning of DGDG formation was concomitant with the end of the transient PC increase that was measured as early as 2 h after transfer in the –P_i medium. The fact that in our experiment we did not detect any transient PC increase in the first hours of P_i deprivation (data not shown) may be explained by the different plant species and in particular by the different system investigated (cell suspension and whole roots).

In bean roots PA increased soon after P_i deprivation (Fig. 2b), indicating its involvement in the early signal transduction of P_i deficiency. After 4 h, the amount of PA was far higher than that of PBut (30.1 and 1.5 mol% of the total PL, respectively). Indeed, while PBut is a relative measure of PLD, PA can be formed also by PLC or synthesised as intermediate in the biosynthesis of glyco- and PL (Ruelland et al., 2002). The different acyl composition of PA in comparison with PLD-derived PBut (Fig. 4) provides further evidence for PA being formed via different pathways. The rapid and transient kinetics of PA is typical of second messengers released by activation of phospholipases. A general property of intracellular signals is that any increase in concentration is transient so that the signal level, after decreasing, can increase again in response to another stimulus (Navari-Izzo et al., 2006). PA is considered a lipid messenger that may activate MAPK signalling cascades, Ca^{2+}-dependent protein kinases, NADPH oxidase complexes, and ion channel activities (Wang, 2002; Navari-Izzo et al., 2006). It was also observed that PA may stimulate root elongation and lateral root formation by mediating signal transduction, membrane trafficking, and/or cytoskletal rearrangements for better nutritional absorption (Li et al., 2006).

In contrast to the previous findings which reported phospholipase activities after long-term P_i deprivation, the accumulation of DAG, together with the enhancement of PBut proportion in −P bean roots after few hours of incubation (Figs. 2a and c), may reflect the induction of different phospholipases involved in signalling the lowered P_i status. It has been observed that the activation of type B MGDG synthases by P_i deprivation was directly triggered by signal transduction via a P_i-sensing mechanism, not by P_i starvation-induced damage to plants (Kobayashi et al., 2006). It is likely that P_i deprivation-induced replacement of PL with DGDG is tightly controlled by P_i signalling. Indeed, in response to a short-term P_i starvation, the level of PA, compound that is now accepted to play a major role in signalling, was early elevated with a very rapid kinetics. In addition, the increase in DAG and PBut suggests the involvement of both PLC and PLD in P_i deficiency signal transduction providing a very exciting field for our future research.

4. Experimental

4.1. Plant material

In the long-term experiment, seeds of *Phaseolus vulgaris* L. (cv Bianco di Bagnasco) were germinated in the dark for four days and the seedlings were transferred to 4 l continuously aerated containers (20 plants in each container) filled either with a phosphate-sufficient (+P) Knop nutrient solution containing 3 mM $Ca(NO_3)_2$, 1.5 mM KNO_3, 1.25 mM $MgSO_4$, 1 mM KH_2PO_4 and 0.04 mM Fe–EDTA or with a phosphate-deficient (−P) Knop medium in which KCl replaced KH_2PO_4. The growth media were changed every 4 days. Plants were grown in controlled conditions under 16/8 h (day/night) photoperiod, with a photon flux density of 400 μmol m^{-2} s^{-1}, 25/20 °C (day/night) temperatures and 75–85% relative humidity. At day 18 roots from intact plants were cut and immediately analysed to evaluate their phosphorous content and PM lipid composition.

In the short-term experiment, plants were grown for 18 days in the same +P nutrient solution and conditions described above. After this period, one set of intact plants was transferred to a −P medium for increasing periods of time (1, 2, 4, 6, 24, 48, 72 and 96 h). For PLD determination the plants were incubated in the −P solution in the presence of 0.2% *n*-butanol. The other set of plants was kept in the + P solution as a control. At each time point, roots from both treatments were harvested and immediately used for analyses.

4.2. Phosphorous content

Roots were dried at 70 °C until constant weight, ground and digested with $HClO_4$. The determination of phosphorous content was carried out following the procedure described by Allen (1940).

4.3. Plasma membrane isolation

The PM of roots was isolated using a two-phase aqueous polymer partition system. Roots were homogenised in an isolation medium consisting of 50 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 3 mM Na_2EDTA, 10 mM ascorbic acid, and 5 mM diethyldithiocarbamic acid. The homogenate was filtered through four layers of a nylon cloth and centrifuged at 10,000*g* for 10 min. The supernatant was further centrifuged at 65,000*g* for 30 min to yield a microsomal pellet, which was resuspended in 2 ml of a resuspension buffer (5 mM K–Pi, pH 7.8, 0.25 M sucrose, and 3 mM KCl). PM was isolated by loading the microsomal suspension (1.0 g) onto an aqueous two-phase polymer system to give a final concentration of 6.5% (w/w) Dextran T500, 6.5% (w/w) polyethylenglycol, 5 mM K–Pi (pH 7.8), 0.25 M sucrose, and 3 mM KCl. The PM was further purified using a two-step batch procedure. The resulting upper phase was diluted 4-fold with 50 mM Tris–HCl (pH 7.5) containing 0.25 M sucrose, and centrifuged for 30 min at 100,000*g*. The resultant PM pellet was resuspended in the same buffer containing 30% ethyleneglycol and stored at −80 °C till lipid analyses. To check the purity of the PM, the activity of the vanadate-sensitive ATPase as a marker enzyme was determined. Cytochrome *c* oxidase, NADH cytochrome *c* reductase and NO_3^--sensitive ATPase activities were used as markers of mitochondria, endoplasmic reticulum and tonoplast, respectively (Navari-Izzo et al., 1993).

4.4. Isolation of plastids

Roots were homogenised in a Waring blender 5 × 3 s in a ice-cold isolation medium (1:10, w/v) containing 0.33 M sucrose, 5 mM ascorbate, 0.2% bovine serum albumin, 50 mM Mes–NaOH (pH 6.6). The homogenate was filtered through four layers of Miracloth and centrifuged at 3000*g* for 5 min. Pellets were subjected to osmotic shock with a solution containing 10 mM Mes–NaOH (pH 6.6), 10 mM NaCl, 2 mM $MgCl_2$ for 10 min in the dark. After centrifugation at 12,000*g* for 5 min, the plastidial membranes were recovered.

4.5. Extraction and analysis of lipids

Lipids were extracted from the PM suspension by the addition of boiling *iso*-PrOH followed by $CHCl_3$-MeOH (2:1, v/v) containing butylhydroxytoluol (50μg ml^{-1}) as an antioxidant. The solvent mixture was then washed with 0.88% KCl to separate the $CHCl_3$ phase. The upper H_2O phase was re-extracted with $CHCl_3$, the $CHCl_3$ phases combined and dried under a stream of N_2. Total lipids were fractionated into neutral lipid, glycolipid and PL fractions on Sep-Pak cartridges (Waters, Milford, MA) (Navari-Izzo et al., 2006). Lipid extracts dissolved in $CHCl_3$–HOAc (100:1, v/v) were transferred to cartridges and sequentially eluted with 20 ml of $CHCl_3$–HOAc (100:1, v/v) for neutral lipids, 10 ml of Me_2CO and 10 ml of Me_2CO–HOAc (100:1, v/v) for glycolipids and 7.5 ml of MeOH–$CHCl_3$–H_2O (100:50:40, v/v/v) for PL. $CHCl_3$ (2.25 ml) and H_2O (3 ml) were added successively to the eluate containing the PL to obtain a phase separation and to facilitate their recovery. The separation of individual lipids was performed by TLC (Silica Gel 60, 0.25 mm thickness; Merck, Damstadt, Germany) with the following solvent mixture: petroleum ether–Et_2O–HOAc (80:35:1, v/v/v) for neutral lipids (free sterols and DAG); $CHCl_3$–MeOH–H_2O (65:25:4, v/v/v) for glycolipids (steryl glycosides and cerebrosides); $CHCl_3$–MeOH–HOAc–H_2O (85:15:10:3.5) for PL. After development, the bands were located with iodine vapour. Individual lipids were identified by co-chromatography with authentic standards. Quantitative analyses of sterols, cerebrosides and PL were performed as reported by Navari-Izzo et al. (1993) using cholesterol, glucose and KH_2PO_4 as standards, respectively.

The molecular species of DGDG recovered from plastids and PM of roots were separated on Silica gel G plates impregnated with 10% (w/v) $AgNO_3$. After being activated for 5 h at 110 °C, cooled and stored in the dark, TLC plates were developed in $CHCl_3$–MeOH–H_2O (65:25:4, v/v/v) and the bands sprayed with 0.1% (w/v) 2′,7′-dichlorofluorescein in 95% EtOH for detection under UV light.

4.6. Fatty acid analysis

The fatty acid methyl ester derivatives were obtained as previously described (Quartacci et al., 1997) and separated by GLC on a Dani 86.10 HT gas chromatograph equipped with a 60 m × 0.32 mm SP-2340 fused silica capillary column (Supelco Sigma–Aldrich, St. Louis) coupled to a flame ionisation detector (column temperature 175 °C). Both the injector and detector were maintained at 250 °C. Nitrogen was used as the carrier gas at 0.9 ml min^{-1} with a split injector system (split ratio 1:100). The double bond index (DBI) was calculated as: [(% trienes × 3) + (% dienes × 2) × (% monoenes)]/∑% saturated fatty acids.

4.7. Phospholipase C and D activities

PLC activity was assayed determining the formation of DAG. After the extraction and separation by TLC of the neutral lipid fraction, DAG were quantified on the basis of their fatty acid content and conversion factor (Quartacci et al., 1997).

PLD activity was measured as the production of *in vivo* PBut essentially as described by Navari-Izzo et al. (2006). Following the extraction and separation of the PL fraction from lipids of whole roots, PBut was isolated from the rest of the PL developing the TLC plates in the organic upper phase of a solvent mixture composed by EtOAc–*iso*-octane–HOAc–H_2O (13:2:3:10). After development, bands were located with iodine vapours and PBut identified by co-chromatography with an authentic standard (Avanti Polar Lipids, Alabaster, AL). Quantitative analysis of PBut was performed determining its phosphorous content as reported by Navari-Izzo et al. (1993) using KH_2PO_4 as standard.

4.8. Statistical analysis

Results are the means of two replicates of three independent experiments ($n = 3$). One-way ANOVA was used for comparisons between means. Means followed by an asterisk are significantly different at $P \leqslant 0.05$.

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