

Lipid Profiling by Electrospray Ionization Tandem Mass Spectrometry and the Identification of Lipid Phosphorylation by Kinases in Potato Stolons

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ABSTRACT: There is limited information about the involvement of lipids and esterified fatty acids in signaling pathways during plant development. The purpose of this study was to evaluate the lipid composition and molecular species of potato (*Solanum tuberosum* L., cv. Spunta) stolons and to identify phosphorylated lipids in the first two developmental stages of tuber formation. Lipid profiling was determined using ESI-MS/MS, a useful method for the determination of the biosynthesis and catabolism of lipids based on their fatty acid composition. The most prevalent compound identified in this study was phosphatidic acid (PA); digalactosyldiacylglycerol (DGDG) was the second most abundant compound. A 34:2 species was identified in PA, phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). The identification of lipid phosphorylation by kinases was revealed by the presence of the phosphorylated lipids. PA was metabolized to diacylglycerol pyrophosphate (DGPP) by phosphatidic acid kinase (PAK). This work establishes a correlation between lipid fatty acid composition and lipid metabolism enzymes at the beginning of tuber formation and is the first report of PAK activity in the early events of potato tuber formation.

KEYWORDS: *Solanum tuberosum*, potato, electrospray ionization tandem mass spectrometry, phospholipid, galactolipid, kinase

■ INTRODUCTION

The potato (*Solanum tuberosum* L.) is one of the world's most important crops and occupies fourth place in the annual production of food for human consumption.¹ In Argentina, the main provinces that produce potatoes are Buenos Aires, Mendoza, Tucumán, and Córdoba.²

The formation of potato tubers begins with the thickening of the stolon tip, which is caused by cell expansion and division.³ The synthesis of structural lipids is essential for cell membrane expansion, and the lipid messengers generated are involved in the transduction of several hormonal and environmental signals.⁴

Glycosylglycerides and phosphoglycerides are the primary lipids in plant membranes. Common plant phospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA). The galactoglycerolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are also present in plants. Among the phosphoglycerides, PC and PE are the primary lipids, and PS is a minor component of most membranes. PG is located in the photosynthetic membranes and the extrachloroplastid membranes; PI and its derivatives are located

in plasma membranes, where they function in signal transduction along with PA.⁵ PA is formed directly via activation of phospholipase D (PLD) and indirectly via the activation of the phospholipase C (PLC) pathway. The action of PLC generates diacylglycerol (DAG) as the first product and DAG is rapidly converted to PA by DAG kinase (DGK).⁶ Turning off the PA signal and removing PA is accomplished by enzymes such as phosphatidic acid kinase (PAK), which phosphorylates PA to produce DGPP.⁷

The lipid kinases phosphatidylinositol (PtdIns) 4-kinase and PtdIns4P 5-kinase, which are present in the plasma membrane, are responsible for the synthesis of the phosphorylated derivatives of PtdIns (PI). The phosphoinositide PtdIns4P (PIP) and PtdIns4,5P₂ (PIP₂), which constitute a minor proportion of total cellular lipids,⁸ regulate cytoskeleton dynamics.⁹ A PI-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂), which produces the second messengers inositol 1,4,5-trisphosphate and DAG.¹⁰

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The major lipid constituents of chloroplasts are MGDG and DGDG.¹¹ While studies have focused on the role of MGDG and DGDG in the photosynthetic process,¹² both lipids have also been identified in nonphotosynthetic tissues such as potato tubers.¹³

Numerous studies have shown that plant galactolipids exhibit antitumor, anti-inflammatory, and antiviral activities.¹⁴ To ensure membrane homeostasis, galactolipid biosynthesis must be regulated during plant development and when environmental conditions change.¹⁵ Nonphotosynthetic plastids possess the potential to synthesize substantial amounts of galactolipid for a variety of uses. The ability of DGDG to substitute for missing phospholipids in the plasma membranes of phosphate-deprived roots as well as the emerging role of galactolipids in the formation of the peribacteroid membrane in legume roots emphasize the importance of further studies concerning galactolipid biosynthesis.¹⁶

Recently, a sensitive approach based on electrospray ionization tandem mass spectrometry (ESI-MS/MS) was employed to profile membrane lipid molecular species, identify the compositional changes in lipids that are associated with plant development,¹⁷ determine the lipid species that serve as the substrate and products of specific enzymes,¹⁸ and identify enzymes of lipid metabolism that are involved in several plant responses.¹⁹ This sensitive method, which uses a triple quadrupole mass spectrometer, has numerous advantages over traditional analytical methods because chromatographic procedures can be avoided, which allows for direct analysis of the biological sample extract.¹⁸ Using ESI-MS/MS, polar lipids in each class of lipids can be identified based on the total number of carbons and the number of double bonds present using precursor or neutral loss scanning of headgroup-specific fragments. Various headgroup scans are conducted sequentially to obtain a complete polar lipid molecular species profile.²⁰

The identification and determination of potato tuber lipids has been reported,¹³ and the loss of selective membrane permeability due to a change in the physicochemical state of the membrane lipids has been shown to occur concurrently with an increase in sugar concentration over the several months of storage at low temperatures²¹ required for tuber sweetening. Additionally, changes in phospholipids and the activation of phospholipases were reported during tuber formation.⁴ However, the lipid signaling mechanism has not been elucidated. There is also little known about the fatty acid composition of phospholipids, galactolipids, and phosphorylated lipids during the onset of tuber formation. The purposes of the present study were to (i) perform lipid profiling in potato stolons using ESI-MS/MS, (ii) determine a possible correlation between fatty acid composition and the involvement of specific lipid metabolism enzymes, and (iii) analyze the involvement of lipid kinases in the early events of tuberization.

MATERIALS AND METHODS

Chemicals. Solvents were purchased from Merck (Darmstadt, Germany). [³²P]γ-ATP (370 MBq mL⁻¹) was obtained from Amersham Pharmacia Biotech (UK). Other chemicals were purchased from Sigma-Aldrich (Argentina).

Preparation of Samples. Mother tubers of potatoes (*Solanum tuberosum* L., cv. Spunta) were sown in a greenhouse with a 15 h photoperiod: 30 °C day/12 °C night. At the time of initiation of the tuberization process (28 days post sowing), the stolons corresponding to the first stage of tuberization (hooked apex stolons) were collected.

Subsequently, stolons corresponding to the second stage of tuberization (initial swelling stolons) were collected. Samples were immediately immersed in liquid nitrogen and lyophilized over three days using a freeze-dry system (Labconco); lyophilized samples were used for ESI-MS/MS lipid profiling. Fresh samples were also prepared by immersion in homogenization buffer for lipid phosphorylation determination.

Lipid Extraction. Lipid extraction was performed as described by Welti et al.¹⁸ For each sample, 10 mg dry weight (dry wt) of stolons was immersed in 3 mL of isopropanol (0.01% w/v butylated hydroxytoluene) at 75 °C for 15 min. Then, 1.5 mL of chloroform and 0.6 mL of distilled water were added. The tubes were shaken for 1 h at room temperature, which was followed by the removal of the extract. The stolons were re-extracted with chloroform/methanol (2:1) with 0.01% butylated hydroxytoluene five times with 30 min of agitation each time until all of the remaining plant tissue appeared white. The combined extracts were washed once with 1 mL of 1 M KCl and once with 2 mL of water. The organic phase was recovered, and the solvent was evaporated under a nitrogen stream. The lipid extracts were sealed in a glass tube with a Teflon tape screw cap and stored at -20 °C until the analysis.

Lipid Identification and Quantification. Lipid identification and quantification were performed by an automated electrospray ionization tandem mass spectrometry approach as previously described¹⁸ with modifications. An aliquot of 5 to 300 μL of sample extract in chloroform was used for ESI-MS/MS, and different amounts of internal standards were added in the following quantities: 0.6 nmol di12:0-PC, 0.6 nmol di24:1-PC, 0.6 nmol 13:0-lysoPC, 0.6 nmol 19:0-lysoPC, 0.3 nmol di12:0-PE, 0.3 nmol di23:0-PE, 0.3 nmol 14:0-lysoPE, 0.3 nmol 18:0-lysoPE, 0.3 nmol di14:0-PG, 0.3 nmol di20:0(phytanoyl)-PG, 0.3 nmol 14:0-lysoPG, 0.3 nmol 18:0-lysoPG, 0.23 nmol 16:0-18:0-PI, 0.16 nmol di18:0-PI, 0.2 nmol di14:0-PS, 0.2 nmol di20:0(phytanoyl)-PS, 0.3 nmol di14:0-PA, 0.3 nmol di20:0(phytanoyl)-PA, 0.49 nmol 16:0-18:0-DGDG, 0.71 nmol di18:0-DGDG, 2.01 nmol 16:0-18:0-MGDG, and 0.39 nmol di18:0-MGDG. The mixture containing the sample and internal standard was combined with chloroform/methanol/300 mM ammonium acetate in water in a ratio of 300/665/35 and a final volume of 1.4 mL. The lipid extracts were introduced into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA) using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland). The identification of the lipid species was performed using the scans previously described¹⁸ with the following modifications: [M + NH₄]⁺ in positive ion mode with neutral loss (NL) 189.0 for PG; [M + NH₄]⁺ in positive ion mode with NL 277.0 for PI; [M + H]⁺ in positive ion mode with NL 185.0 for PS; [M + NH₄]⁺ in positive ion mode with NL 115.0 for PA; [M + NH₄]⁺ in positive ion mode with NL 341.1 for DGDG; and [M + NH₄]⁺ in positive ion mode with NL 179.1 for MGDG. The scan speed was 50 or 100 μm per s. The collision energies were +40 V for PC, +28 V for PE, +20 V for PG, +25 V for PI, PS, and PA, +24 V for DGDG, and +21 V for MGDG.

Three replicates were analyzed by mass spectrometry. The data were isotopically deconvoluted and the molecular species quantified using the internal standards of the same lipid class. The quantification, expressed as nmol mg⁻¹ dry wt, from all three replicates was averaged and used to calculate the standard deviations. The data were transformed with a natural logarithm for statistical analysis using ANOVA and the Tukey posteriori test.

Lipids Phosphorylation by Kinases. Samples of 500 mg of stolon in the first or second stage of tuberization were ground to powder with mortar and pestle in liquid N₂ and added to 3 mL of homogenization buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM KCl, and 250 mM saccharose) with 30 μL of protease inhibitors. After 15 min of centrifugation at 3300 rpm at 4 °C, the liquid supernatant was collected, and the pellet debris was re-extracted with an additional 500 μL of homogenization buffer. The resulting supernatant was then centrifuged at 33000 rpm for 45 min to obtain the membrane fraction, which was resuspended in 100 μL of 50 mM HEPES at pH 7.4. For the determination of phosphorylated lipids by endogenous kinases

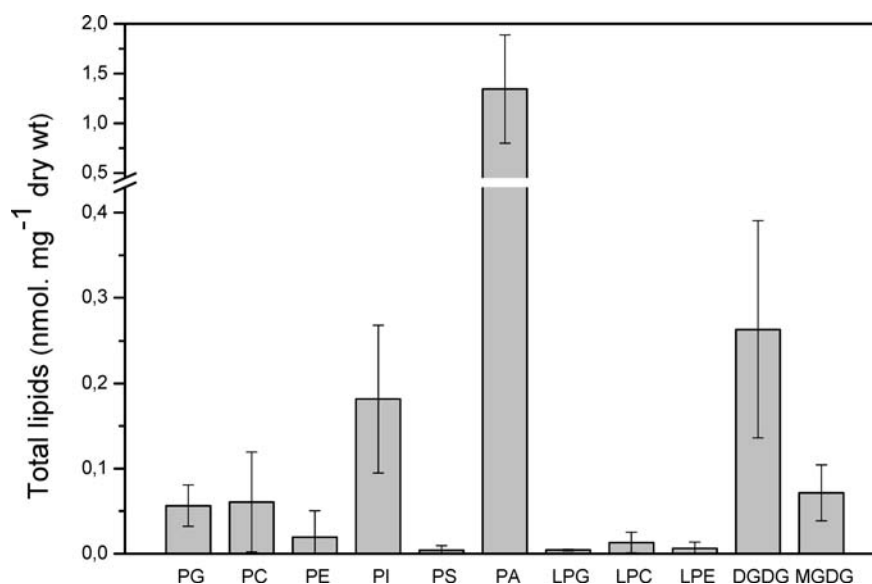


Figure 1. Total lipid content of potato stolons at the initiation of tuber formation. The analysis was performed by ESI-MS/MS on three independent extracts. The results are expressed as nmol mg^{-1} dry wt representing the mean \pm SE. The lipids quantified were phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid, lysophosphatidylglycerol (LPG), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), digalactosyldiacylglycerol (DGDG), and monogalactosyldiacylglycerol (MGDG).

activity, 60 μg of protein from the membrane fraction obtained above was added to 30 $^{\circ}\text{C}$ buffer containing 200 mM HEPES, pH 7.4, 10 mM sodium ortho-vanadate, 200 mM EDTA, 2.4 M MgCl_2 , 50 μM DTE, and $[^{32}\text{P}]\gamma\text{-ATP}$ (3.7×10^6 Bq). The reaction was stopped after 2 min by adding 1.5 mL of chloroform/methanol (1/2, v/v), followed by 0.5 mL of 2.4 N HCl and 0.5 mL of chloroform. Phosphorylated lipids were extracted from the membranes²² and then separated by TLC.²³ The position of each radiolabeled phosphorylated lipid was determined by autoradiography on Kodak film. Resulting spots were scraped from the plates and counted in a scintillation counter.

Four replicates of each stage of tuber formation were analyzed. The values were averaged and expressed as pmol/min/mg protein.

RESULTS

ESI-MS/MS Lipid Profiling. The phospholipids, lysophospholipids and galactolipids present in potato stolons were quantified by ESI-MS/MS (Figure 1). Because this method identifies individual species for each lipid class (phospholipid, lysophospholipid, and galactolipid), the total content was calculated by adding the amounts for all of the species of each lipid (expressed as the nmol mg^{-1} dry wt of total lipids present) in potato stolons for three independent experiments.

The lipid composition in potato stolons was 82.5% phospholipids, 16.5% galactolipids, and 1.0% lysophospholipids. There are six phospholipids in potato stolons that were quantified by ESI-MS/MS; the primary phospholipid present in potato stolons was PA, which accounted for 67.3% of the total lipid content ($1.35 \text{ nmol mg}^{-1}$ dry wt). The second most abundant phospholipid was PI (8.94%), which was followed by PG (2.85%), PC (2.59%), PE (0.66%), and PS (0.14%). The lysophospholipids included lysophosphatidylcholine (LPC, 0.57%), lysophosphatidylethanolamine (LPE, 0.25%), and lysophosphatidylglycerol (LPG, 0.23%). Among the galactolipids, digalactosyldiacylglycerol (DGDG) was the most abundant (12.9%), followed by monogalactosyldiacylglycerol (MGDG, 3.5%) (Figure 1).

An example of the ESI-MS/MS spectrum corresponding to the molecular ions of the phospholipid PA, lysophospholipids

LPC/PC and galactolipid DGDG of potato stolons with the internal standards for each lipid class is shown in Figure 2. The molecular species of each lipid class were identified by the ion mass. For example, the sample spectrum for PA showed a peak at 690.6 m/z corresponding to 34:2-PA and a peak at 714.5 m/z corresponding to 36:4-PA.

Fatty Acid Profiling. ESI-MS/MS lipid profiling identified lipid species in terms of their headgroup class, total carbon number, and total number of double bonds.²⁰ The lysophospholipids quantified in potato stolons included palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), long chain fatty acids (>C18), and diacyl lipids (Table 1). The lipids in potato stolons consisted of a complex mixture of fatty acids. The main combinations of fatty acids in each class could be one of the following:¹⁴ C16:0/C18:2 (34:2), C16:0/C18:3 (34:3), C16:3/C18:3 (34:6), C18:00/C18:2 (36:2), C18:0/C18:3 (36:3), C18:1/C18:3 (36:4), C18:2/C18:3 (36:5), and C18:3/C18:3 (36:6). This list includes galactolipids that occur in edible plants, but the list does not imply positional information about the fatty acids on the glycerol.

The most common acyl species quantified were 34:2 (40.16%), followed by 36:4 (20.96%), 34:3 (11.30%), 36:5 (8.78%), 36:2 (6.01%), 36:6 (4.29%), 36:3 (2.87%), 32:0 (2.40%), 34:1 (0.71%), 34:0 (0.57%), 18:2 (0.52%), and 16:0 (0.48%). There were additional species in this group at the lower levels listed in Table 1. The acyl species with percentages below 0.01% are not shown in Table 1.

Lipid Molecular Species. Among the molecular species found in potato stolons, PA was the predominant lipid (Figure 3 and Table 1); the most abundant acyl chains were 34:2 (29.37%), followed by 36:4 (17.25%), 34:3 (6.70%), 36:5 (5.87%), and 36:2 (4.32%). The main molecular species of PI were also the 34:2 (5.93%) and 34:3 (1.85%) acyl chains. PC was primarily found as 34:2 (1.42%) and 36:4 (0.42%), and the percentage of acyl chain of PS was lowest with respect to the

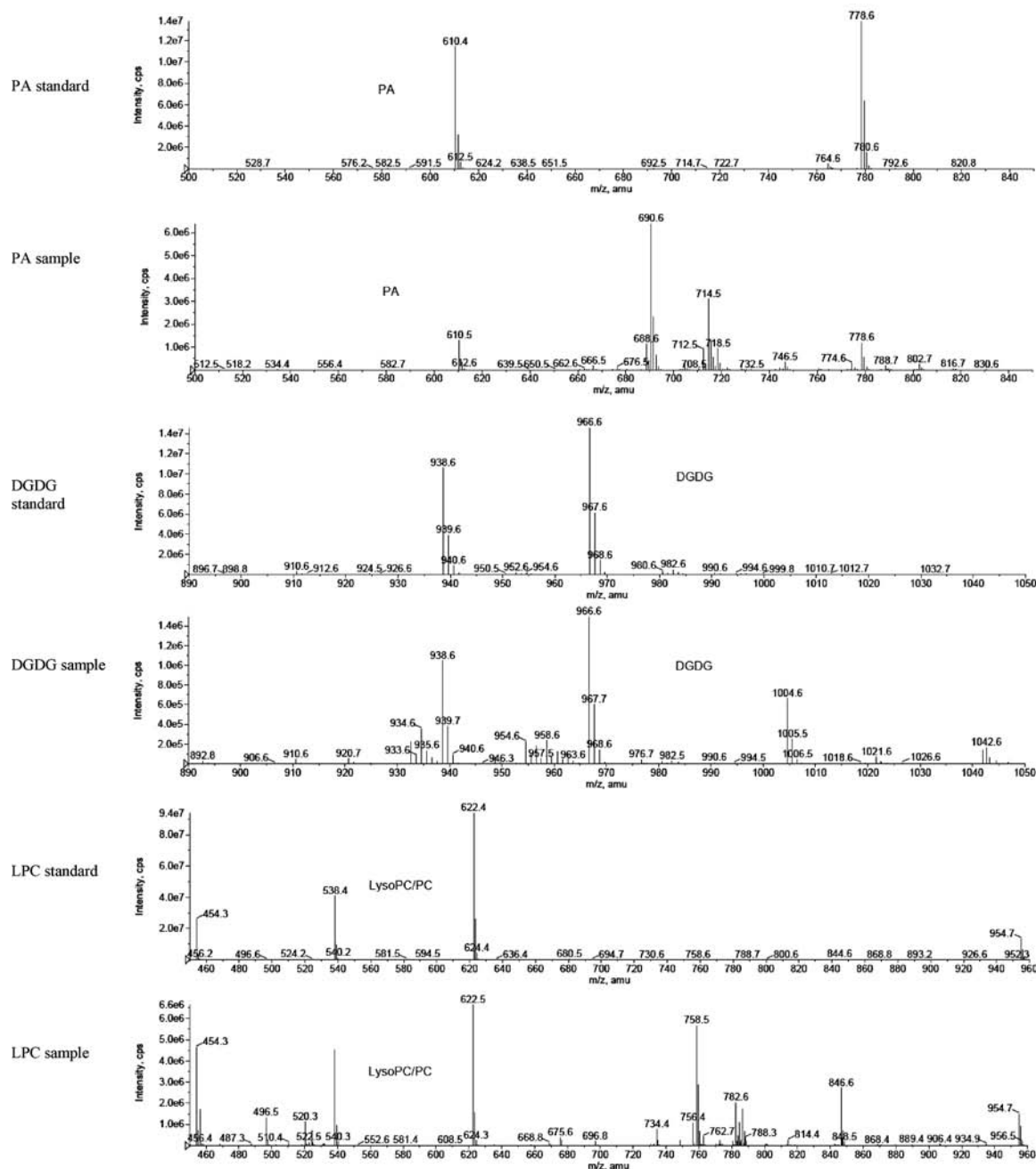


Figure 2. ESI-MS/MS spectra corresponding to molecular ions of the phospholipid PA, the lysophospholipid PC, and the galactolipid DGDG of potato stolons relative to internal standards. ESI-MS/MS precursor scans of mass-to-charge ratio (m/z) were 610.4 (di14:0-PA), 778.6 (di20:0(phytanoyl)-PA), 938.6 (16:0–18:0-DGDG), 966.6 (di18:0-DGDG), 454.3 (13:0-lysoPC), and 538.4 (19:0-lysoPC).

other phospholipids, with a majority found as 38:2 (Table 1 and Figure 3).

The lysophospholipids were detected at the lowest levels (Table 1). LPC was the most abundant lysophospholipid, with 18:2 as the main acyl chain present, followed by the 16:0 chain. LPE was primarily observed in 16:0 chains, whereas LPG was present as 18:2 chains.

We found DGDG to be the main galactolipid present in the potato stolons (Table 1 and Figure 4). The samples contained the polyunsaturated acyl species 36:6 (2.87%), followed in decreasing order of abundance by 34:2 (2.38%), 34:3 (2.08%), and 36:4 (1.85%). The main molecular species of MGDG were 36:6 (1.0%) and 36:4 (1.0%), followed by 36:5 (0.85%).

Lipid Phosphorylation by Kinases. To determine which phospholipids were phosphorylated in the first two developmental stages of tuber formation, enrichment with [32 P] γ -ATP and detection through two-dimensional separation of phospholipids were used. The phospholipid analysis revealed the presence of PIP₂, PIP, DGPP, LPA, and PA (Figure 5). The quantification of each TLC spot revealed that the primary lipid phosphorylated in the first stage of development, expressed in units of pmol/min/mg protein, was DGPP (12.08), followed by LPA (2.94), PIP (2.19), PIP₂ (1.21), and PA (1.19). During the second stage of tuberization, the amounts of all the phospholipids decreased: DGPP to 6.71, LPA to 1.60, PIP to 1.71, PIP₂ to 0.78, and PA to 0.65. Although this trend was observed for all phospholipids, the changes in PA, DGPP, and

Table 1. Acyl Species of the Lipid Molecular Species in Potato Stolons^a

acyl chain	total	percentage of acyl species in each lipid molecular species													
		PA	PI	PG	PC	PE	PS	LPC	LPE	LPG	DGDG	MGDG			
16:0	0.48							0.25	0.20	0.03					
16:1	0.03							0.01		0.02					
18:0	0.06							0.06							
18:1	0.06							0.03		0.03					
18:2	0.52							0.27	0.11	0.14					
18:3	0.04							0.04							
32:0	2.40	0.60	0.06	1.65	0.09										
32:1	0.02		0.02												
32:2	0.01		0.01												
34:0	0.57		0.57												
34:1	0.71	0.34	0.01	0.06	0.04									0.26	
34:2	40.16	29.37	5.93	0.38	1.42	0.43	0.01							2.38	0.24
34:3	11.30	6.70	1.85	0.07	0.30	0.08								2.08	0.22
34:4	0.12	0.08	0.01											0.02	0.01
34:5	0.03													0.02	0.01
34:6	0.13													0.01	0.12
36:1	0.05		0.01		0.01									0.02	0.01
36:2	6.01	4.32	0.52	0.01	0.34	0.08	0.02							0.69	0.03
36:3	2.87	1.48	0.17	0.13		0.03	0.01							0.99	0.06
36:4	20.96	17.25	0.29	0.04	0.42	0.11								1.85	1.00
36:5	8.78	5.87	0.10	0.01	0.15	0.04								1.76	0.85
36:6	4.29	0.40	0.01		0.01									2.87	1.00
38:2	0.16				0.05	0.04	0.07								
38:3	0.08				0.01	0.01	0.03							0.03	
38:4	0.02													0.02	
38:5	0.01													0.01	
40:2	0.13				0.03	0.07	0.03								
40:3	0.01					0.01									
42:2	0.06					0.06									
42:3	0.02					0.01	0.01								
Total	100	66.4	9.6	2.4	2.9	1.0	0.2	0.7	0.3	0.2	13.0	3.5			

^aThe results are expressed as a percentage of acyl species in each lipid molecular species.

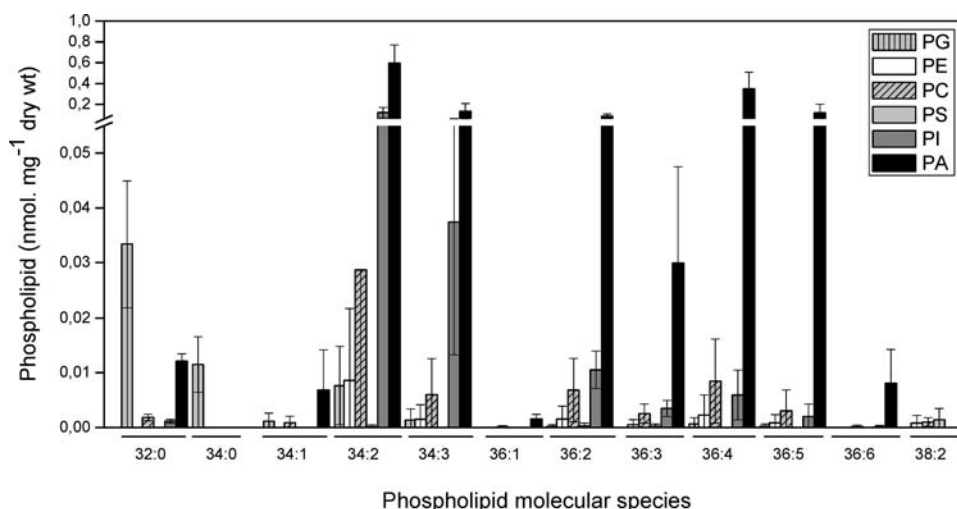


Figure 3. Phospholipid molecular species at the initiation of potato tuber formation. The analysis was performed by ESI-MS/MS on three independent extracts. The results are expressed as nmol mg^{-1} dry wt representing the mean \pm SE. Note the differences in the scales of PI and PA relative to the other phospholipids.

LPA levels were most significant; they decreased to approximately half the initial values. The kinase assay was used to determine the PA content that accumulated as a result

of the action of DGK. The results of the kinase assay indicated that part of the total PA found in the stolon was present due to the activity of DGK, which phosphorylated DAG, leading to the

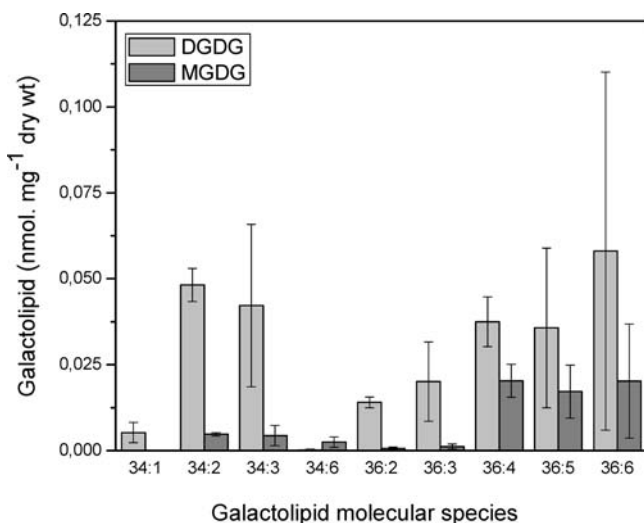


Figure 4. Galactolipid molecular species at the initiation of potato tuber formation. The analysis was performed by ESI-MS/MS on three independent extracts. The results are expressed as nmol mg⁻¹ dry wt representing the mean \pm SE.

product PA. Similarly, the DGPP detected was the result of the phosphorylation of PA by a phosphatidic acid kinase (PAK), LPA could be produced by the activation of PA-PLA, and PIP and PIP₂ were the products of PtdIns 4-kinase and PtdIns4P 5-kinase, respectively.

DISCUSSION

The present study has determined the lipid profiling of potato stolons using ESI-MS/MS, determined a possible correlation between fatty acid composition and the involvement of specific lipid metabolism enzymes, and demonstrated the involvement of lipid kinases revealed by the presence of the phosphorylated lipids PIP₂, PIP, DGPP, LPA, and PA.

Phospholipids are formed through the activation of phospholipases and lipid kinases. Among plant phospholipids, PA is an essential lipid precursor for phospholipids and galactolipids and has an established role as a lipid second messenger. In roots, PA is involved in the early signal transduction of P_i deficiency²⁴ and has also been shown to function in the stimulation of root elongation and lateral root formation.²⁵ However, the complete role of PA in plant development is still emerging.²⁶

Phospholipase A₁ (PLA₁), phospholipase A₂ (PLA₂), PLD, and PLC have been identified as lipid hydrolyzing enzymes.²⁷ In a previous report, PLD and PLC were found to be activated in potato stolons at an early stage of tuber formation, and a high PA level was observed in stolons. Exogenous PA caused an increase in the stolon cell area of central mother cells and rib meristem cells.⁴ Thus, according to this previous research, PA is an important signal that mediates the role of jasmonic acid (JA) in the initiation of tuber formation through changes in the lipid species.

Our study, through the employment of a novel and sensitive method (ESI-MS/MS), corroborates that PA constitutes a major percentage of total lipids. The PA levels in this study are similar to those found by Cenzano et al.⁴ in potato stolons using a traditional lipid analytical method as well as the levels observed in pea root plastids¹⁶ and sunflower seeds.²⁸ Furthermore, we found similar percentages of PI, PS, DGDG, and MGDG to those reported in potato tubers by Galliard.¹³

PA is the product of DAG phosphorylation catalyzed by DAK.²⁹ PA can be metabolized during signal transduction through the production of diacylglycerol pyrophosphate (DGPP) by phosphatidic acid kinase (PAK).⁷ PA can also be transformed to LPA by PLA³⁰ to decrease signaling. We observed that potato stolons also have the ability to undergo the phosphorylation of phospholipids through the action of lipid kinases. In the first stage of tuber formation, we found DGPP at high levels, indicating that PA was metabolized to DGPP by PAK.

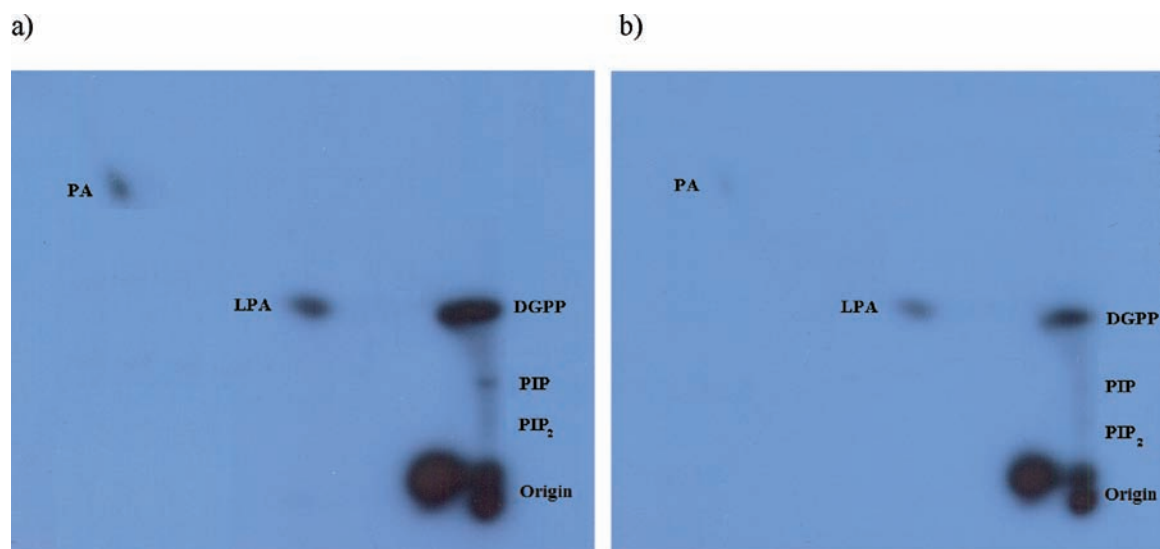


Figure 5. Two-dimensional separation of phosphorylated lipids from the first (a) and second (b) stage of potato tuberization. Total membrane fractions were incubated at 30 °C for 2 min in the presence of [³²P]γ-ATP. The phosphorylated lipids were extracted and separated by two-dimensional TLC, using the following solvent systems: (1) chloroform/methanol/acetone/acetic acid/H₂O (40:14:15:12:7, v/v) and (2) pyridine/chloroform/formic acid (15:17.5:3.5, v/v). The identification of each lipid was carried out by comparison of their retention factor with those of commercial standards.

The high level of PA found, which is produced by PLD, PI-hydrolyzing phospholipase C (PI-PLC), and DGK activities, is necessary for cell expansion at the initiation of tuber formation.⁴ Once initiation of tuber formation is complete, the signal is attenuated.

Phosphatidylinositol (PI) is another phospholipid involved in signal transduction. The plant phosphoinositide system is involved in the regulation of key physiological processes that are important for plant function and development, such as polar growth of pollen tubes and root hairs or guard cell function.³¹ The high level of PI found in this study reveals that this phospholipid, together with PA, is involved in the signal transduction that occurs during tuber formation. Moreover, this work demonstrates that phosphoinositides also have an important role during tuber formation as substrates of the phosphorylation cascade in which PIK is the first active kinase.

A nonspecific lipolytic acylhydrolase (LAH) present in tubers of solanaceous plants is a patatin that has combined PLA₁ and PLA₂ activity. A patatin protein with PLA₂ activity has been proposed to be involved in the biosynthesis of JA by providing linolenic acid substrate.³² Although high levels of LAH activities were found in tubers of *Solanum tuberosum* L.,³³ we found a low level of lysophospholipids and free fatty acids in potato stolons. It has been suggested that overexpressors of patatin-like genes do not display acute phenotypes that would be expected from massive membrane degradation.³⁴ Therefore, patatin-like enzymes are likely to have a post-translational activation mechanism, and this type of regulation is likely to occur for enzymes involved in cell signaling processes.³⁵ However, we found high amounts of LPA during the kinase assay in first stage stolons, suggesting that this lysophospholipid could have been produced as a result of the action of a PLA over PA.

Galactolipids are important food constituents in both animals and humans and are expected to be an important source of essential fatty acids.³⁶ Galactolipids are also present in non-photosynthetic tissues such as potato tubers, apples, and seed grains; in potato tubers, 14.2% of the lipids are DGDG, and 5.7% are MGDG.¹³ In agreement with Galliard et al.,¹³ we found that the amount of phospholipids in stolons consistently exceeds the amount of galactolipids. Galactolipids predominate in the amyloplast, and an increase of these lipids in the membrane of this plastid could help maintain fluidity and permeability in an attempt to avoid starch breakdown.³⁷ However, a role for these compounds during tuber formation has not been elucidated.

Nongreen plastids generally have low levels of 16:3 fatty acids in their galactolipids, and even galactolipids isolated from *Arabidopsis* roots (normally a 16:3 plant) did not contain 16:3 fatty acids.³⁸ Consistent with this information, we were not able to detect 16:3 free fatty acids in potato stolons.

In potato tubers, the phospholipids PC and PE of the 18:2/18:2 species are predominant.³⁹ However, in potato stolons we found 34:2 PA (18:2/16:0) to be the most abundant species, followed by 36:4 PA (18:2/18:2).

Taking into account the proportions of similar PA species found among the lipids analyzed, it was possible to distinguish the PA produced by PLD or DGK coupled to the activation of PI-PLC. In the potato plant, three PI-PLC isoforms were present in various tissues.¹⁰ Our data suggest that 34:2 PA was formed primarily by phosphorylation of DAG through DGK prior to the activation of PI-PLC, as revealed by the high level of PI and the presence of the phosphoinositide substrates. However, the production of PA through PLD activity was

minimally important, as revealed by the lower levels of the membrane phospholipids PC and PE, which are substrates of PLD. In addition, 34:2 PA formation from galactolipids (hydrolysis of MGDG and phosphorylation of DAG) was not observed. Thus, evaluation of the PA molecular species formed at the initiation of tuber formation provides further information about the PA formation pathway.

Galactolipids are characterized by a very high content of polyunsaturated fatty acids, mostly 18:3 (linolenic acid) and, to a lesser extent, 16:3 (hexadecatrienoic acid).⁴⁰ The high proportion of 18:3/18:3 in the 36:6-DGDG and 36:6-MGDG species found in our study suggests that hydrolysis could release the 18:3 substrate for the biosynthesis of JA, a promoter of tuberization,^{41–43} as previously suggested.⁴⁴

Another area of concern is that plastids from 18:3 plants are thought to have insufficient PA phosphatase activity to generate the required levels of endogenous DAG to support de novo galactolipid biosynthesis.⁴⁰ However, the promotion of de novo fatty acid synthesis and glycerolipid assembly in pea root plastids provides sufficient amounts of PA precursor to support the synthesis of MGDG at these levels.¹⁶ In contrast to the results of McCune et al.,¹⁶ we observed a different fatty acid composition between phospholipids and galactolipids, which led us to conclude that galactolipids do not contribute to PA production through DAG and the subsequent phosphorylation by DGK, as was observed during freezing.⁴⁵ Therefore, there must be an independent origin of MGDG and DGDG from PA. In this way, galactolipids could be synthesized through the eukaryotic lipid pathway by MGDG and DGDG synthases.

In summary, this study demonstrates a lipid profile specifically associated with the initiation of tuber formation, establishes a correlation between lipid fatty acid composition and lipid metabolism enzymes, and is the first report of phosphatidic acid kinase (PAK) involvement in early events of potato tuber formation. ESI/MS-MS is a useful method for the determination of the biosynthesis and catabolism of lipids based on their fatty acid composition. For this reason, application of this technique to a developmental process such as tuberization may be helpful for understanding how different pathways for phospholipid metabolism are involved in the stolon-to-tuber potato transition. Further studies are required for a deeper understanding of the changes in lipid metabolism and its respective roles during the tuberization process (from stolon formation to tuber development) and the importance of potato tuber quality for human consumption.

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■ ABBREVIATIONS USED

ABA, abscisic acid; ESI-MS/MS, electrospray ionization tandem mass spectrometry; DAG, diacylglycerol; DGDG, digalactosyl-diacylglycerol; DGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; A₁, JA, jasmonic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PAK, phosphatidic acid kinase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP₂, phosphatidylinositol-4,5-bisphosphate; PS, phosphatidylserine

■ REFERENCES

- (1) Fernie, A. R.; Willmitzer, L. Molecular and biochemical triggers of potato tuber development. *Plant Physiol.* **2001**, *127*, 1459–1465.
- (2) Caldiz, D. O.; Haverkort, A. J.; Struik, P. C. Analysis of a complex crop production system in interdependent agro-ecological zones: a methodological approach for potatoes in Argentina. *Agric. Syst.* **2002**, *73*, 297–311.
- (3) Vreugdenhil, D.; Struik, P. C. An integrated view of the hormonal regulation of tuber formation in potato (*Solanum tuberosum* L.). *Physiol. Plant.* **1989**, *75*, 525–531.
- (4) Cenzano, A.; Cantoro, R.; Racagni, G.; De los Santos-Briones, C.; Hernández-Sotomayor, S. M. T.; Abdala, G. Phospholipid and phospholipase changes by jasmonic acid during stolon to tuber transition of potato. *Plant Growth Regul.* **2008**, *56*, 307–316.
- (5) Harwood, J. L. Plant Lipid Metabolism. In *Plant Biochemistry*; Dey, P. M., Harborne, J. B., Eds.; Academic Press: London, U.K., 1997; pp 237–272.
- (6) Arisz, S. A.; Testerink, C.; Munnik, T. Plant PA signaling via diacylglycerol kinase. *Biochim. Biophys. Acta* **2009**, *9*, 869–875.
- (7) Munnik, T.; Vrije, T.; Irvine, R.; Musgrave, A. Identification of diacylglycerol pyrophosphate as a novel metabolic product of phosphatidic acid during G-protein activation in plants. *J. Biol. Chem.* **1996**, *271*, 15708–15715.
- (8) Drøbak, B. K. The plant phosphoinositide system. *Biochem. J.* **1992**, *288*, 697–712.
- (9) Wasteneys, G. O.; Galway, M. E. Remodeling the cytoskeleton for growth and form: an overview with some new views. *Annu. Rev. Plant Biol.* **2003**, *54*, 691–722.
- (10) Kopka, J.; Pical, C.; Gray, J. E.; Müller-Röber, B. Molecular and enzymatic characterization of three phosphoinositide-specific phospholipase C isoforms from potato. *Plant Physiol.* **1998**, *116*, 239–250.
- (11) Allen, C. F.; Good, P. Plant lipids. *J. Am. Oil. Chem. Soc.* **1965**, *42*, 610–614.
- (12) Jarvis, P.; Dörmann, P.; Peto, C. A.; Lutes, J.; Benning, C.; Chory, J. Galactolipid deficiency and abnormal chloroplast development in the *Arabidopsis* MGD synthase 1 mutant. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8175–8179.
- (13) Galliard, T. Aspects of lipid metabolism in higher plants. I. Identification and quantitative determination of the lipids in potato tubers. *Phytochemistry* **1968**, *7*, 1907–1914.
- (14) Christensen, L. P. Galactolipids as potential health promoting compounds in vegetable foods. *Recent Pat. Food, Nutr. Agric.* **2009**, *1*, 50–58.
- (15) Kelly, A. A.; Öhman, A.; Sedoud, A.; Wieslander, Å. Membrane Homeostasis Mechanisms for Plant Glycosyl-Transferases Involved in Galactolipid Biosynthesis: Biochemical and in-Silico Structure Studies of Key Enzymes. In *Current Advances in the Biochemistry and Cell Biology of Plant Lipids*; Benning, C., Olhrogge, J., Eds.; Aardvark Global Publishing Company: Salt Lake City, UT, 2007; pp 25–31.
- (16) McCune, L. M.; Kleppinger-Sparace, K. F.; Sparace, S. A. Monogalactosyldiacylglycerol Synthesis in Pea Root Plastids. In *Current Advances in the Biochemistry and Cell Biology of Plant Lipids*. Benning, C., Olhrogge, J., Eds.; Aardvark Global Publishing Company: Salt Lake City, UT, 2007; pp 32–37.
- (17) Fauconnier, M. L.; Welti, R.; Blée, E.; Marlier, M. Lipid and oxylipin profiles during aging and sprout development in potato tubers (*Solanum tuberosum* L.). *Biochim. Biophys. Acta* **2003**, *1633*, 118–126.
- (18) Welti, R.; Li, W.; Li, M.; Sang, Y.; Biesiada, H.; Zhou, H.-E.; Rajashekar, C. B.; Williams, T. D.; Wang, X. Profiling membrane lipids in plant stress responses: Role of phospholipase D α in freezing-induced lipid changes in *Arabidopsis*. *J. Biol. Chem.* **2002**, *277*, 31994–32002.
- (19) Nandi, A.; Krothapalli, K.; Buseman, C. M.; Li, M.; Welti, R.; Enyedi, A.; Shah, J. The *Arabidopsis thaliana* sfd mutants affect plastidic lipid composition and suppress dwarfing, cell death and the enhanced disease resistance phenotypes resulting from the deficiency of a fatty acid desaturase. *Plant Cell.* **2003**, *15*, 2383–2398.
- (20) Welti, R.; Wang, X. Lipid species profiling: a high-throughput approach to identify lipid compositional changes and determine the function of genes involved in lipid metabolism and signaling. *Curr. Opin. Plant Biol.* **2004**, *7*, 1–8.
- (21) Wismer, W. V.; Worthing, W. M.; Yada, R. Y.; Marangoni, A. G. Membrane lipid dynamics and lipid peroxidation in the early stages of low-temperature sweetening in tubers of *Solanum tuberosum*. *Physiol. Plant.* **1998**, *102*, 396–410.
- (22) Schacht, J. Extraction and Purification of Polyphosphoinositides. In *Methods in Enzymology*; Lowenstein, J. M., Ed.; Academic Press: New York, 1981; Vol. 72, pp 626–631.
- (23) Racagni-Di Palma, G.; Brito-Argáez, L.; Hernández-Sotomayor, S. M. T. Phosphorylation of signaling phospholipids in *Coffea arabica* cells. *Plant Physiol. Biochem.* **2002**, *40*, 899–906.
- (24) Russo, M. A.; Quartacci, M. F.; Izzo, R.; Belligno, A.; Navari-Izzo, F. Long- and short-term phosphate deprivation in bean roots: Plasma membrane lipid alterations and transient stimulation of phospholipases. *Phytochemistry* **2007**, *68*, 1564–1571.
- (25) Li, M.; Welti, R.; Wang, X. Quantitative profiling of *Arabidopsis* polar glycerolipids in response to phosphorus starvation. Roles of phospholipases D ζ 1 and D ζ 2 in phosphatidylcholine hydrolysis and digalactosyldiacylglycerol accumulation in phosphorus-starved plants. *Plant Physiol.* **2006**, *142*, 750–761.
- (26) Testerink, C.; Munnik, T. Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. *J. Exp. Bot.* **2011**, *62*, 2349–2361.
- (27) Wang, X.; Wang, C.; Sang, Y.; Qin, C.; Welti, R. Networking of phospholipases in plant signal transduction. *Physiol. Plant.* **2002**, *115*, 331–335.
- (28) Regente, M.; Corti Monzón, G.; de la Canal, L. Phospholipids are present in extracellular fluids of imbibing sunflower seeds and are modulated by hormonal treatments. *J. Exp. Bot.* **2008**, *59*, 553–562.
- (29) Munnik, R. T.; Irvine, R. F.; Musgrave, A. Phospholipid signalling in plants. *Biochim. Biophys. Acta* **1998**, *1389*, 222–272.
- (30) Wang, X. Lipid signaling. *Curr. Opin. Plant Biol.* **2004**, *7*, 329–336.
- (31) Heilmann, I. Using genetic tools to understand plant phosphoinositide signaling. *Trends Plant Sci.* **2008**, *14*, 171–179.
- (32) Senda, K.; Yoshioka, H.; Doke, N.; Kawakita, K. A cytosolic phospholipase A2 from potato tissues appears to be patatin. *Plant Cell Physiol.* **1996**, *37*, 347–353.
- (33) Galliard, T. Enzymic deacylation of phospholipids and galactolipids in plants. Purification and properties of a lipolytic acyl hydrolase from potato tubers. *Biochem. J.* **1971**, *121*, 379–390.
- (34) La Camera, S.; Geoffroy, P.; Samaha, H.; Ndiaye, A.; Rahim, G.; Legrand, M.; Heitz, T. A pathogen-inducible patatin like lipid acyl hydrolase facilitates fungal and bacterial host colonization in *Arabidopsis*. *Plant J.* **2004**, *44*, 810–825.
- (35) Matos, A. R.; Pham-Thi, A. T. Lipid deacylating enzymes in plants: old activities, new genes. *Plant Physiol. Biochem.* **2009**, *47*, 491–503.

(36) Andersson, L.; Bratt, C.; Arnoldsson, C. K.; Herslof, B.; Olsson, N. U.; Sternby, B.; Nilsson, A. Hydrolysis of galactolipids by human pancreatic lipolytic enzymes and duodenal contents. *J. Lipid Res.* **1995**, *36*, 1392–1400.

(37) Liljenberg, C.; Sandelius, A. S.; Selstam, E. Effect of storage in darkness and in light on the content of membrane lipids of potato tubers. *Physiol. Plant.* **1978**, *43*, 154–159.

(38) Awai, K.; Maréchal, E.; Block, M. A.; Brun, D.; Masuda, T.; Shimada, H.; Takamiya, K.; Ohta, H.; Joyard, J. Two types of MGDG synthase genes, found widely in both 16:3 and 18:3 plants, differentially mediate galactolipid syntheses in photosynthetic and nonphotosynthetic tissues in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10960–10965.

(39) Demandre, C.; Tremolieres, A.; Justin, A.-M.; Mazliak, P. Analysis of molecular species of plant polar lipids by high-performance and gas liquid chromatography. *Phytochemistry* **1985**, *24*, 481–485.

(40) Joyard, J.; Teyssier, E.; Miège, C.; Berny-Seigneurin, D.; Maréchal, E.; Block, M. A.; Dorne, A.-J.; Rolland, N.; Ajlani, G.; Douce, R. The biochemical machinery of plastid envelope membranes. *Plant Physiol.* **1998**, *118*, 715–723.

(41) Cenzano, A.; Vigliocco, A.; Kraus, T.; Abdala, G. Exogenously applied jasmonic acid induces changes in apical meristem morphology of potato stolons. *Ann. Bot.* **2003**, *91*, 917–921.

(42) Cenzano, A.; Vigliocco, A.; Miersch, O.; Abdala, G. Hydroxylated jasmonate levels during stolon to tuber transition in *Solanum tuberosum* L. *Potato Res.* **2005**, *48*, 107–115.

(43) Cenzano, A.; Abdala, G.; Hause, B. Cytochemical immunolocalization of allene oxide cyclase, a jasmonic acid biosynthetic enzyme, in developing potato stolons. *J. Plant Physiol.* **2007**, *164*, 1449–1456.

(44) Conconi, A.; Miquel, M.; Browse, J. A.; Ryan, C. A. Intracellular levels of free linolenic and linoleic acids. Increase in tomato leaves in response to wounding. *Plant Physiol.* **1996**, *111*, 797–803.

(45) Wang, X.; Devaiah, S. P.; Zhang, W.; Welti, R. Signaling functions of phosphatidic acid. *Prog. Lipid Res.* **2006**, *45*, 250–278.