Chapter 16

METABOLOMIC ANALYSIS OF LOW PHYTIC ACID MAIZE KERNELS

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Abstract: Phytic acid, or hexaphosphorylated *myo*-inositol, is the major storage form of phosphorous (P) in maize kernels. Phytic acid in foods or animal feeds can complex with proteins and mineral cations resulting in reduced bioavailablility of important nutrients. Classic mutation breeding has been used to develop maize plants that produce kernels with significantly less phytic acid. An extensive survey of the low phytate phenotype in different maize genetic backgrounds grown in five field locations revealed that an increase in inorganic P correlated with a decrease in phytic acid P, but the increased amount of inorganic P did not consistently account for the P reduction noted in the low phytate lines. There were no quantitative phosphorous differences in phospholipids or starch. In follow-up experiments using a metabolomics approach, both mutant and wild type kernels were obtained from a single segregating ear, minimizing variability. Individual mature kernels were lyophilized and ground. Kernel phenotype was determined by using a simple colorimetric test for inorganic P content. Kernels of similar phenotype were pooled and extracted in aqueous methanol and partitioned into polar and nonpolar fractions. Metabolites were derivatized and subjected to GC/TOF/MS, and raw data was processed using the Leco ChromaTof peak deconvolution software. Compounds were identified *via* coelution and/or mass spectrum matching with authentic standards. Each of these metabolites was semi-quantified by calculating the ratio of the peak area of a characteristic extracted ion against that of the internal standard and correcting for sample weight. P-containing metabolites were recognized easily by a prominent m/z 299. Several P-containing metabolites were more abundant in low phytic acid kernels, although it is unlikely that they are responsible for the reduced yield associated with this phenotype.

1 INTRODUCTION

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate, Figure 16-1) is a very abundant molecule in the seeds of many cereals and legumes (Shi et al.,

B.J. Nikolau and E. Syrkin Wurtele (eds.), Concepts in Plant Metabolomics, 221–238.

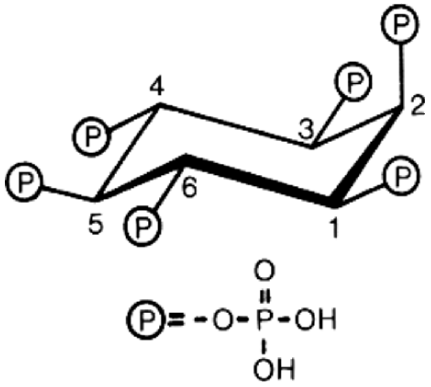

Figure 16-1. Phytic acid, or hexaphosphorylated *myo*-inositol.

2003). The negatively charged molecule is associated *in planta* with cations such as K^+, Mg^{2+}, and Ca^{2+}. As such, phytic acid is a major storage form of *myo*-inositol, phosphate as well as, several mineral cations, all needed to sustain seedling development. Phytic acid is also believed to be central to the control of inorganic phosphate levels in both developing seeds and growing seedlings (Strother, 1980).

The unique chemical properties of phytic acid have significant consequences for human and animal nutrition. Phytic acid absorption by the digestive track depends largely on microbial phytase activity, which is essentially lacking in nonruminant animals, including humans (Holm, 2002). Furthermore, the intact phytic acid molecule will interfere with absorption of nutritionally important minerals such as iron, zinc, and magnesium, resulting in suboptimal animal weight gain, or affect adversely human nutrition in communities dependent on a high grain diet (Zhou and Erdman, 1995). For this reason, phytic acid can be categorized as an anti-nutritional component in many maize-based foods and feeds. Of great concern to the livestock industry is the potential for substantial amounts of phosphorous (P) in the form of undigested phytate to be excreted in animal manure, contributing to environmentally damaging levels of P in runoff from high density livestock operations (Cromwell and Coffey, 1991). Clearly, reducing the amount of seed phytic acid in cereal grains commonly fed to animals and/or people while maintaining the amount required for normal seed and seedling development could be advantageous.

To address these needs, mutant and transgenic low phytic acid maize, barley, rice, wheat, and soybean have been developed (Larson et al., 1998; Wilcox et al., 2000; Hitz et al., 2002; Raboy, 2002; Guttieri et al., 2004). Low phytic acid (*lpa1*) maize is a chemically (EMS) induced mutant with a

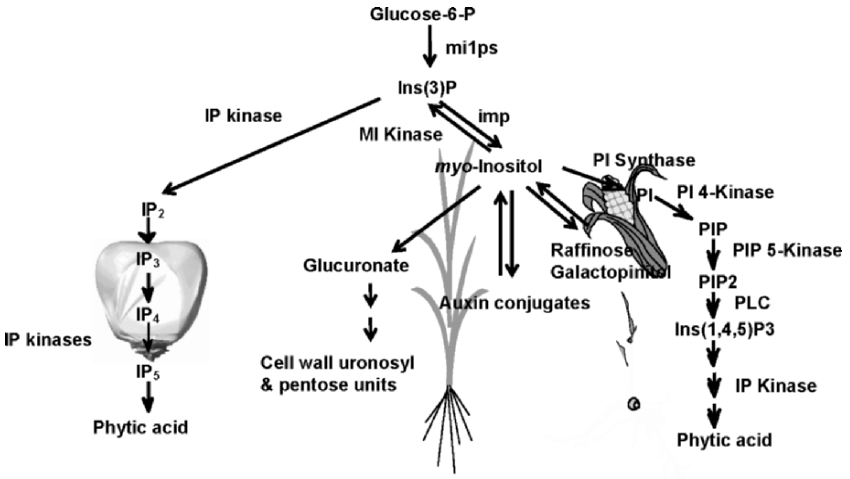

Figure16-2. Putative phytic acid biosynthesis in maize. mi1ps, 1D-*myo*-inositol-1-phosphate synthase; Ins(3)P, *myo*-inositol-3-phosphate; Ins(1,4,5)P3, *myo*-inositol-1,4,5-triphosphate; IP, *myo*-inositol-phosphate; IP_2, diphosphorylated *myo*-inositol-phosphate; IP_3, triphosphorylated *myo*-inositol-phosphate; IP_4, tetraphosphorylated *myo*-inositol-phosphate; IP_5, pentaphosphorylated *myo*-inositol-phosphate; imp, *myo*-inositol monophosphatase; MI, *myo*-inositol; PI, phosphatidylinositol; PIP, phosphatidylinositol-phosphate; PIP2, phosphatedylinositol-diphosphate.

65% reduction in seed phytic acid content and about a tenfold increase in free inorganic phosphorous (Pi). The *myo*-inositol phosphates with fewer than six esterified phosphates ("lower *myo*-inositol phosphates") do not accumulate. We have determined that the *lpa1* mutation essentially does not change the amounts of total P, oil, protein, starch, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{3+}. Although *lpa1* seed development, seed desiccation, seed germination, seedling development, and seedling vigor are all normal, there is typically up to 15% unexplainable loss of kernel dry weight (yield).

The *LPA1* gene has been mapped to Chromosome 1 (Raboy et al., 2000) but its function is not known. Other low phytic acid maize traditional mutants have been isolated subsequently. Kernels of the *lpa2* mutant accumulate significant amounts of *myo*-inositol-P_3, *myo*-inositol-P_4, and *myo*-inositol-P_5 (Shi et al., 2003). A third mutant (*lpa3*), like *lpa1*, does not accumulate the lower myo-inositol phosphates in their seeds (unpublished observation).

The genetics and biochemistry of phytic acid synthesis in maize is incompletely understood despite intensive analyses of several *lpa* mutants (Raboy et al., 2000; Shi et al., 2003). It is clear that the first step in committed phytic acid biosynthesis is the conversion of glucose-6-P to *myo*-inositol-3-P catalyzed by *myo*-inositol-P synthase (Milps, Figure 16-2). In

developing kernels, phytic acid is synthesized by sequential kinase-catalysed phosphorylations. *Myo*-inositol-P can also be dephosphorylated and the liberated sugar alcohol can be incorporated into phosphatidylinositol followed by an alternative phosphorylation pathway to phytic acid. *Myo*-inositol is also the precursor to various raffinosaccharides, cell wall components, and auxin conjugates. However, the metabolic flow through these pathways in wild type and mutant kernels is unknown.

We hypothesize that phosphorous-containing metabolite(s) that accumulate in low phytic acid maize kernels are associated with reduced kernel dry weight. A targeted analysis of phosphorylated compounds was done initially to identify those that might be tied to reduced yield. This effort was followed up with a more comprehensive metabolomics approach. We also anticipate that comparison of results from wild type and low phytic acid type might define better our incomplete understanding of phytic acid biosynthesis in these mutant kernels.

2 METHODS

2.1 Analysis of phytic acid, inorganic, and total P in field-grown seeds

Twenty inbred and hybrid lines with wild-type phenotypes were planted alongside their *lpa1* conversions at five locations within the US Midwest Corn Belt. Plots at each location were harvested at the same time, and the seeds were cleaned, dried, and analyzed for phytic acid, inorganic P, and total P. Phytic acid was measured by anion exchange HPLC. Seeds were ground using a Kleco ball mill (Visalia, CA). Samples were weighed (500 mg) into 20 mL scintillation vials. A 5 mL of 0.4M HCl was added and the samples were shaken on a gyratory shaker at room temperature for 2 h. Extracts were filtered through a 0.45 μm PVDF syringe filter attached to a 5 mL plastic disposable syringe barrel. A 450 μL aliquot was filtered through a 0.2 μm microcentrifuge spin filter unit and then transferred to a 2 mL glass autosampler vial fitted with a 400 μL glass insert. A Dionex DX 500 HPLC equipped with a Thermo Separation Products AS3500 autosampler was used. Extracts were injected in 25 μL amounts onto a Dionex OmniPac^{TM} PAX-100 analytical column (4 × 250 mm) in line with an OmniPac^{TM} PAX-100 guard column (4 × 50 mm) and an ATC-1 anion trap column. A Dionex conductivity detector module II was used with an anion self-regenerating suppressor (ASRS-Ultra II) set up in the external water regeneration mode and operated with a current of 300 mA. Phytic acid was eluted at 1 mL min^{-1} with the following mobile phase program: H_2O/200 mM NaOH/50% aqueous *iso*-propanol (68/30/2) for 4.0 min, followed by a step gradient to H_2O/200 mM

NaOH/50% aqueous *iso*-propanol (39/59/2) at 14.1 min, then a step gradient return to initial conditions at 15.1 min, followed by equilibration for 15 min. The separation was performed at room temperature. The concentration of phytic acid P was calculated from the concentration of phytic acid by dividing the former by the molecular weight of the sodium phytate standard, multiplying by 6 (P per phytic acid molecule), and multiplying by 31 (molecular weight of P). Inorganic P was measured spectroscopically using modifications of the method of Chen et al. (1956) (Shi et al., 2003). Total P was determined by a contract laboratory using inductively coupled plasma spectroscopy.

2.2 Targeted analysis of P-containing kernel constituents

Bulk samples of mature kernels from Pioneer Hybrid 3730 (wild type) and its *lpa1* hybrid conversion were used for targeted analysis of P-containing constituents. These two seed sources were grown at the same field location. Phospholipids were extracted twice from 1.0 g ground seeds in two 3 mL aliquots of ice cold methanol:chloroform:formic acid (10:10:1) with centrifugation for 5 min at 2500 revolutions per minute (rpm) after each extraction. The pellet was re-extracted twice with two 3 mL aliquots of methanol:chloroform:H_2O (5:5:1), again with centrifugation for 5 min at 2500 rpm after each extraction. The supernatants from all four extractions were combined, and 3.6 mL of a solution containing 1.16 mL 85% H_3PO_4 and 7.455 g KCl in a total volume of 100 mL were added. The solution was vortexed and centrifuged for 5 min at 2500 rpm. Major phospholipids in the lower layer were determined by normal phase HPLC with evaporative light scattering detection adapting the method of Picchioni et al. (1996).

Phosphorous was measured in starch extracted from isolated endosperm, the kernel tissue where the majority of starch is found (Perry, 1988). The extraction and purification of starch was according to the method of Bechtel and Wilson (2000) with modifications. The endosperm was ground to a fine powder in a Kleco ball mill. One and one-half grams ground endosperm were incubated for 60 min at 37°C with 25 mL H_2O and 10 mL 0.8% Pepsin A in 0.04N HCl. Five milliliters 0.08% hemicellulase (1500 units/g activity) in 0.1M sodium acetate were added, and the reaction mix was incubated an additional 3 h at 45°C. Five milliliters of detergent mix (5% Triton X-100, 5% Tween 40, 5% SDS, and 5% Triton X-15) were added, and the reactions were vortexed and centrifuged for 5 min at 2500 × g at 20°C, and the supernatant was discarded. The pellet was resuspended in 25 mL H_2O, vortexed, centrifuged, and decanted as before. The water washing, vortexing, centrifuging, and decanting steps were repeated three times. The pellet was resuspended in 2 mL H_2O and applied on a 53 μm or 75 μm-mesh screen and washed with approximately six volumes of water. The filtrate was

centrifuged for 5 min at 2500 × g at 20°C, and the supernatant was discarded. The resulting pellet was dissolved in 3 mL 70% ethanol, vortexed, centrifuged, and the supernatant was discarded. The final purified starch pellet was lyophilized for a minimum of 48 h. The entire procedure was performed on 20 wild type and 20 *lpa1* 1.5 g samples, and the purified starch from each phenotype was pooled to accumulate sufficient material for subsequent total P determination.

2.3 Metabolomic analysis of individual kernels

Wild type and *lpa1*or *lpa3* plants were crossed to obtain kernels on F1 ears segregating 1:1 for the mutant genotype. At physiological maturity, individual kernels were removed from the cob and frozen immediately in liquid nitrogen. Only kernels from the central portion of the ear were harvested; the butt and tip kernels were discarded. The kernels were lyophilized and ground to a fine powder in a Genogrinder 2000 ball mill (SPEX CertiPrep, Metuchen, NJ). The low phytic acid phenotype of individual kernels was determined indirectly by measuring the amount of Pi spectroscopically using modifications of the method of Chen et al. (1956) (Shi et al., 2003).

Metabolites were extracted from 30 mL ground material from each of 10 wild type and 10 low phytic acid kernels. Extraction and chemical derivatization were performed according to the method of Fiehn et al. (2000) (Figure 16-4). Both *lpa1* and *lpa3* mutants and their wild-type controls were analyzed. The unique experimental design minimizes greatly the environmental influence on metabolite expression, since every sample developed within the same ear. The nonpolar fractions, after methylation and trimethylsilylation, and the polar fractions, after methoxyamination and trimethylsilylation, were subjected to GC/TOF/MS. The trimethylsilyl derivatives were separated by gas chromatography on a Supelco 30 M × 0.25 mm I.D. × 0.25 mm film thickness SPB-50 column. One-microliter injections were made with a 1:10 split ratio using an Agilent 7683 autosampler. The polar extracts were rerun in the splitless mode in order to improve sensitivity for some phosphorous-containing metabolites.

An Agilent 6890N gas chromatograph was programmed for an initial temperature of 70ºC for 5 min, increased to 310°C at 5º min^{-1} where it was held for 1 min. The injector and transfer line temperatures were 230ºC and 250ºC, respectively, and the source temperature was 200ºC. He was used as the carrier gas with a constant flow rate of 1 mL min^{-1} maintained by electronic pressure control. Mass spectra were obtained online with a Leco Pegasus III time-of-flight (TOF) mass spectrometer. An electron beam of –70eV was

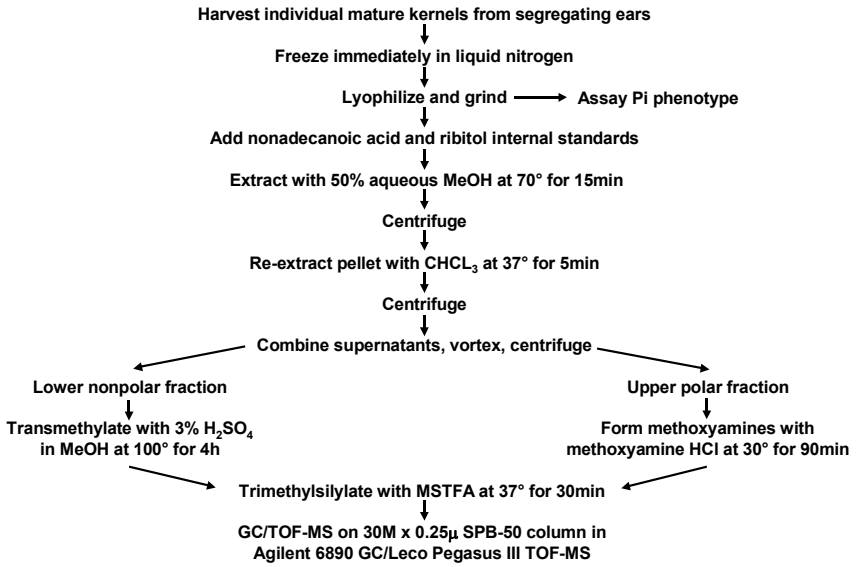

Figure 16-3. Extraction and derivatization scheme for metabolomics analysis of maize kernels. MeOH, methanol; $CHCL_3$, chloroform; MSTFA, N-methyl-N-trimethylsilyl-trifluoro-acetamide.

used to generate spectra with a mass range of m/z 41–999 at a sampling rate of 5 spectra s^{-1}. Metabolites were identified based on a match to both the mass spectrum and retention of appropriately derivatized authentic standards. The relative amount of each metabolite was based on the hand-curated area of the extracted ion chromatogram of a characteristic quantifying m/z value. All quantifications were normalized to the peak area of quantifying m/z value of the internal standard and the initial sample dry weight. Student's T-tests were performed to evaluate the statistical significance of the mean relative amounts of each metabolite in wild type and low phytic acid kernels.

3 RESULTS AND DISCUSSION

3.1 Phosphorous balance in field-grown seeds

The reduced phytic acid content of the *lpa1* mutant was evident in all combinations of genetic background and planting location (Figure 16-4). There were no significant effects of either genetics or location on this relationship. There was about twice the amount of phytic acid in *lpa1*

kernels of sample 20 compared with those of the other genetic backgrounds, but still significantly less than in sample 20 wild-type kernels. This increase is undoubtedly due to the high oil character of sample 20. A proportionally larger embryo characterizes these high oil kernels. Thus, more phytic acid is to be expected on a whole-kernel weight basis since it accumulates preferentially in the embryo (O'Dell et al., 1972). We did not measure phytic acid in isolated embryos. As expected, Pi contents in these kernels exhibited an inverse relationship to that of phytic acid. Total P was relatively constant in kernels of samples 1–19, but was slightly higher in those of the high oil type (sample 20) due to their proportionally larger embryos (Figure 16-4).

The lack of variability seen in measured phytate and Pi of the low phytic acid phenotype observed in this field trial suggests that this trait could be used potentially in a breeding program. However, significant reductions in seed yield are observed consistently with *lpa1* plants compared with wild-type plants. Although the physiological basis for this yield reduction is unknown, an obvious suggested cause would be a disrupted P balance. Yield reduction could be attributed to the reduction in phytic acid and/or elevation in Pi, although it is possible that other P-containing metabolites are involved. Our data show about 32% of the total P in *lpa1* kernels is unaccounted for vs 9% in the wild-type kernels (Table 16-1). There was a very consistent and significant increase in the amount of organic P not associated with phytic acid in low phytic acid kernels in all genetic backgrounds and planting locations. To better understand where the unaccounted P has accumulated, we attempted to quantify P in the major P-containing biomolecules in *lpa1* and wild-type kernels using a targeted analysis approach. For practical reasons, we used a single seed source for this more in-depth analysis.

3.2 Targeted analysis of P-containing kernel constituents

As expected, all four of the major membrane-associated phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine) were found in the nonpolar kernel extracts. However, there were no significant differences in the amounts of any of these phospholipids between wild type and *lpa1* kernels, suggesting that altered membrane function is not associated with reduced *lpa1* kernel weight. We also did not find a significant difference in the amount of P associated with endosperm starch between wild type and *lpa1* kernels. This is perhaps not surprising since a mutation affecting P incorporation into phytic acid in the embryo would influence P content of starch in the endosperm. Although scenarios can be suggested to account for this, it is more likely that the effect of the *lpa1* mutation would be restricted to P metabolism in the embryo. To

investigate this possibility, we plan to measure the P content of embryo-associated proteins. Regardless, the lack of an obvious candidate for the unaccounted P in *lpa1* kernels led us to conduct a more comprehensive metabolomics approach.

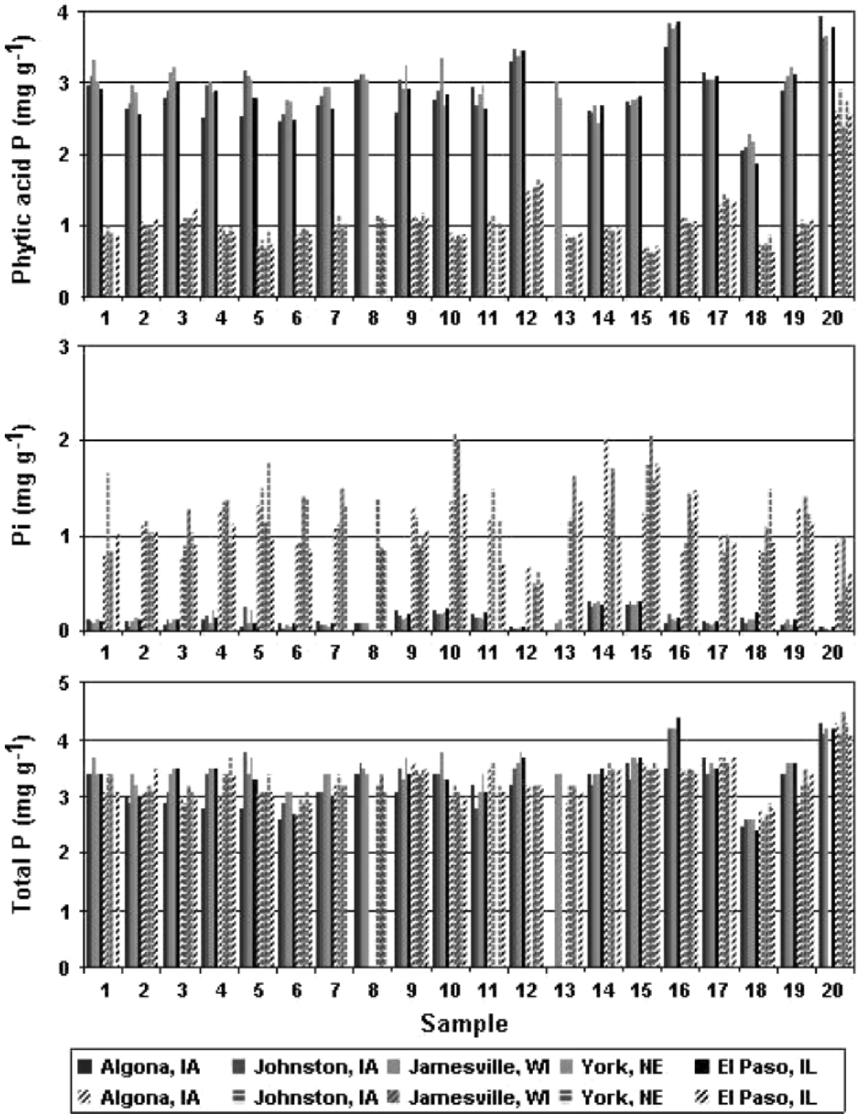

Figure 16-4. Phytic acid phosphorus, Pi, and total P in whole kernels from wild-type plants (solid bars) and their *lpa1* conversions (hashed bars) of different genetic backgrounds grown at five different locations within the US Midwest.

Table 16-1. Phosphorus accounting in wild type and *lpa1* maize kernels

Component	Wt	*lpa1*
	mg g^{-1}	
Total P	3.38	3.25
Phytic acid P	2.93	1.11
Inorganic P	0.14	1.11
Remainder	0.31	1.03

3.3 Metabolomic analysis of individual kernels

Total ion chromatograms from the polar extraction of wild type and *lpa1* kernels were fairly similar (Figure 16-5). Approximately 24 clearly defined peaks were apparent in both samples, with relatively few quantitative differences between the two. However, the high data collection rate of the TOF analyzer coupled with the uniformity of mass spectra across a peak affords automated peak deconvolution, resulting in reliable identification and reproducible quantitation of even very closely eluting metabolites. Total ion chromatograms from the nonpolar extraction of wild type and *lpa1* kernels were also fairly similar to each other (data not shown).

Since we are interested particularly in P-containing metabolites, we took advantage of the fact that m/z 299 in our electron impact mass spectra is diagnostic of such compounds due to instability of the ester-linked trimethylsilylphosphate moiety. This allowed us to identify likely sinks for the unaccounted P in lpa1 kernels, even if these molecules exist at very low relative abundances. Not surprisingly, most of the peaks characterized by an m/z 299 fragment were significantly more abundant in lpa1 kernels compared with wild-type kernels (Figure 16-6). These peaks include the TMS derivatives of phosphoric acid, glycerol-3-phosphate, phosphatidylinositol, and two unknown phosphorylated metabolites (Table 16-2). In addition, an unknown metabolite of phosphatidylinositol, defined as such since it was identified when an authentic phosphatidylinositol standard was subjected to the sample preparation and derivation process, was significantly more abundant in wild-type kernels. However, all of the organic P-containing metabolites were present at low levels in *lpa1* kernels, too low to account for

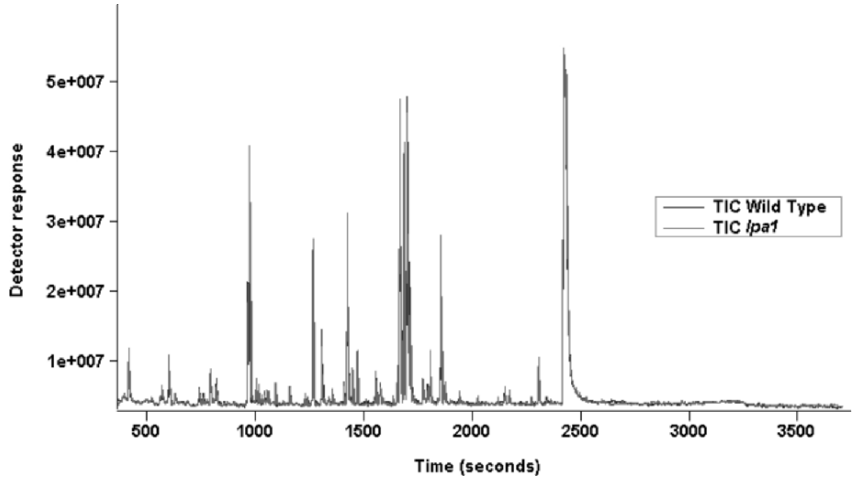

Figure 16-5. Total ion chromatograms (TIC) of polar extractions of wild type and *lpa1* kernels.

a significant proportion of the unaccounted P. Interestingly, our findings are not consistent with the presumed biosynthetic pathway of phytic acid in *lpa1* mutant kernels. This may not be surprising since we worked with fully mature kernels that (1) should exhibit much less active phytic acid synthesis than developing kernels and (2) contain a significant amount of endosperm that does not accumulate phytic acid, thus effectively diluting metabolites involved directly in phytic acid synthesis. For these reasons, we plan to repeat this study with developing embryos.

The relative expression of all the identified polar and nonpolar metabolites in *lpa1* compared with wild-type kernels is presented in Tables 16-2 and 16-3, respectively. There were only two metabolites, glycerol and phosphoric acid, that were found in both polar and nonpolar extracts. Both metabolites were far more abundant in the polar fraction, as expected for molecules with multiple hydroxy groups. Their presence in the nonpolar fraction could be due to hydrolysis of hydrophobic glycerolipids and phospholipids during sample preparation, probably during the high temperature methylation step. Intact glycerolipids or phospholipids were not found in the nonpolar metabolite profiles since they are not detectable with the GC conditions employed. Regardless, it is important to recognize such supposed artifacts of the analytical process when evaluating the biological significance of metabolite profiles. As expected, metabolites represented by two different derivatives (i.e., different numbers of methyl or trimethylsilyl groups) showed similar expression between the two phenotypes, indicating consistent extraction and detection. Aside from the aforementioned P-containing metabolites, the *lpa1* phenotype was associated with little change

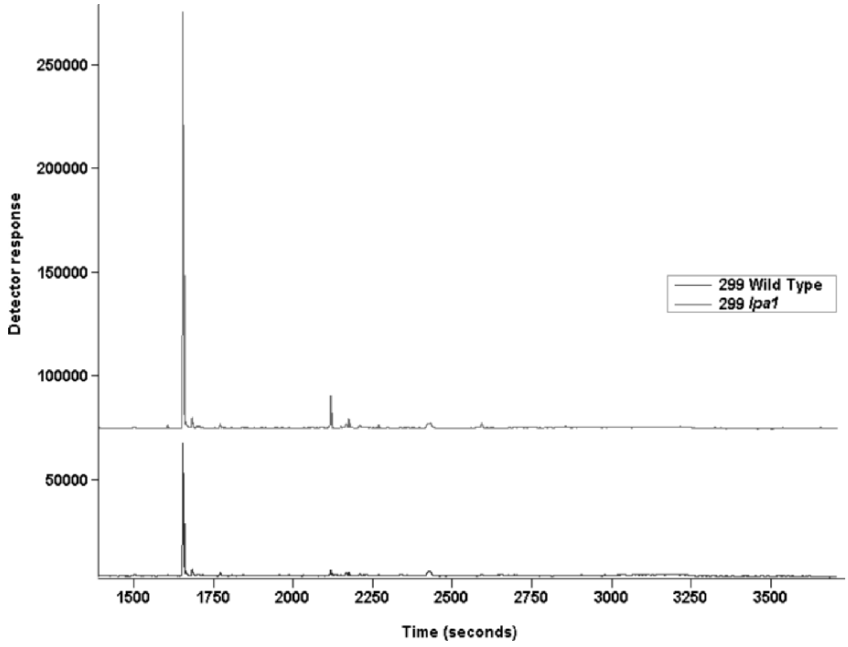

Figure 16-6. Partial extracted ion chromatograms for m/z 299 of polar extractions of wild type and *lpa1* kernels.

in the amounts of the primary metabolites measured. This result suggests either a limited perturbation of primary metabolism by the *lpa1* mutation, or alternatively, the analytical precision and number of replicate samples were insufficient to uncover more subtle changes. A somewhat different picture emerges from the relative expression of identified polar and nonpolar metabolites in *lpa3* kernels compared to their wild-type controls (Tables 16-4 and 16-5, respectively). As in *lpa1* kernels, the relative amounts of several P-containing metabolites were correlated with the *lpa3* genotype. These metabolites were phosphoric acid, glycerol-3-phosphate, sucrose-6-phosphate, phosphatidylinositol, and all three unknown phosphorylated metabolites observed in *lpa1* kernels. As in *lpa1* kernels, all of the organic P-containing metabolites in *lpa3* kernels were present at levels too low to account for a significant portion of the unaccounted P. As in *lpa1* kernels, our results are not consistent with the presumed biosynthetic pathway of phytic acid in *lpa3* kernels. We also plan to repeat this study with developing embryos.

The metabolic profile of *lpa3* kernels exhibits far more differences in non-P-containing metabolites compared to wild type than that of *lpa1*

kernels. Several amino acids, organic acids, sugars, fatty acids, fatty alcohols, alkanes, phenolic acids, and phytosterols were affected. However, the physiological significance of the altered amounts of these diverse primary metabolites is unknown. Several phenolic acids were found in both polar and nonpolar extracts, often as different derivatives. For example, the TMS ester, TMS ether derivatives of caffeic and ferulic acids were found in the polar fraction, while the methyl ester, TMS ether derivatives appeared in the nonpolar fraction that underwent transmethylation. Although there were

Table 16-2. Differential polar metabolite expression in *lpa1* and wt maize kernels

Metabolite[a]	*lpa1*:wt	Metabolite [a]	*lpa1*:wt
Alanine,N,O TMS	0.9	Malic acid TMS	1.1
β-Alanine,N,N,O TMS	0.8	Succinic acid TMS	1.1
4-Aminobutyric acid TMS	0.5**	Glycerol-3-phosphate TMS	1.5**
Asparagine,N,N,O TMS	1.0	*myo*-Inositol-1/3-phosphate TMS	1.3
Asparagine,N,N,N,O TMS	0.5	Phosphatidylinositol TMS	1.7**
Asparatic acid,N,O,O TMS	0.7*	Phosphatidylinositol metabolite TMS	0.8**
Glutamic acid,N,O,O TMS	0.9	Phosphoric acid,O,O,O TMS	1.8***
Glutamine,N,N,O TMS	1.0	Unknown phosphorylated metabolite TMS 1	4.3***
Glycine,N,N,O TMS	0.8	Unknown phosphorylated metabolite TMS 2	1.8**
Homoproline,O TMS	0.9	Arabinitol TMS	1.2
Homoproline,N,O TMS	0.9	Erythritol TMS	0.8
2-Hydroxyglutaric acid TMS	1.2	myo-Inositol TMS	1.7**
Isoleucine,N,O TMS	1.0	Sorbitol TMS	0.6
Leucine,N,O TMS	0.9	Caffeic acid TMS	b
Methionine,N,O TMS	0.8	Ferulate acid TMS	0.2
Phenylalanine,N,O TMS	1.0	Adenine TMS	1.5
Proline,N,O TMS	0.9	Arabinose MeOX2 TMS	0.9
Pyroglutamic acid,N,O TMS	0.9	Fructose MeOX1 TMS	0.8*
Serine,O,O TMS	0.0	Fructose MeOX2 TMS	0.8*
Serine,N,O,O TMS	0.9	Galactose MeOX1 TMS	0.5
Threonine,N,O,O TMS	0.7	Galactose MeOX2 TMS	2.0
Tyrosine,N,O TMS	0.8	Glucose MeOX1 TMS	0.8
Tyrosine, N,O,O TMS	1.0	Glucose MeOX2 TMS	0.8
Valine,N,O TMS	1.0	Glucuronic acid MeOX1 TMS	0.9
Glyceric acid TMS	1.0	Glucuronic acid MeOX2 TMS	b
Glycerol TMS	0.9	Raffinose TMS	0.8
Citric acid TMS	0.9	Sucrose TMS	1.0
Fumaric acid TMS	0.7		

[a]TMS, trimethylsilyl ester; MeOX, methoxyamine.
[b]Not found in wild type.
*Means are significantly different at P<0.1.
**Means are significantly different at P<0.05.
***Means are significantly different at P<0.01.

Table 16-3. Differential nonpolar metabolite expression in *lpa1* and wild type maize kernels

Metabolite[a]	*lpa1*:wt
14:0 Me	1.2
16:0 Me	1.0
16:0 TMS	1.2
16: 1cisΔ7 Me	1.1
17:0 Me	1.1
18:0 Me	1.1
18:1cisΔ9 Me	1.0
18:1cisΔ9 TMS	0.8
18:2cisΔ9,12 Me	1.0
18:2cisΔ9,12 TMS	1.6
18:3cisΔ9,12,15 Me	0.9
20:0 Me	1.5
20:1cisΔ11 Me	2.0
22:0 Me	0.4
23:0 Me	0.8
24:0 Me	1.1
25:0 Me	3.7
26:0 Me	2.6
2HO-20:0 MeTMS	1.3
2HO-22:0 MeTMS	2.2
2HO-24:0 MeTMS	0.5
ρ-Coumaric acid MeTMS	0.7
3,5-Di-tert-butyl-4-hydroxybenzoic acid Ee	1.2**
4-Methoxy, 3-hydroxycinnamic acid Me	1.1
Campesterol TMS	0.6
β-Sitosterol Me	1.1
β-Sitosterol TMS	1.2
Stigmasterol TMS	0.2
Glycerol TMS	b
Phosphoric acid,O,O,O TMS	0.3

[a]Me, methyl ester; TMS, trimethylsilyl ester; MeTMS, methyltrimethylsilyl ester; Ee, ethyl ester.
[b]Not found in *lpa1*.
**Means are significantly different at $P<0.05$.

some free phenolic acids in the polar fraction, as a class they were far more abundant in the nonpolar fraction. This finding suggests that the phenolic acids were liberated by hydrolysis of hydrophobic conjugates, presumably phenolic acids esterified to phytosterols and/or various acyl groups, during sample preparation, most likely during transmethylation. As with glycerol and phosphoric acid in *lpa1* kernels, the biological significance of phenolic

Table 16-4. Differential polar metabolite expression in *lpa3* and wild type maize kernels

Metabolite[a]	*lpa3*:wt	Metabolite [a]	*lpa3*:wt
β-Alanine,N,N,O TMS	0.9	Indoleacetic acid TMS	0.7
ρ-Coumaric acid MeTMS	1.0	Homoproline,N,O TMS	0.2**
2-Hydroxyglutaric acid TMS	1.7**	Isoleucine,N,O TMS	0.7**
4-Aminobutyric acid TMS	1.2	Leucine,N,O TMS	0.6***
4-Hydroxybenzoic acid TMS	0.9	Lysine,N,N,N',O TMS	1.2
5-Hydroxyindoleacetic acid TMS	1.0	Malic acid TMS	0.9
5-Hydroxynorvaline,N,O,O TMS	1.1	Methionine,N,O TMS	0.6**
Adenine TMS	1.0	*myo*-Inositol TMS	1.4
Alanine,N,O TMS	0.9	*myo*-Inositol-1/3-phosphate TMS	1.2
Arabinitol TMS	0.6	Ornithine,N,N,N',O TMS	1.0
Arabinose MeOX2 TMS	1.2	Ornithine,N,N,O TMS	1.2
Asparagine,N,N,N,O TMS	0.8	Phenylalanine,N,O TMS	0.8
Asparagine,N,N,O TMS	1.0	Phenylalanine,O TMS	0.9
Asparatic acid,N,O,O TMS	0.7*	Phosphatidylinositol metabolite TMS	1.5**
Benzoic acid TMS	1.1	Phosphatidylinositol TMS	16.4***
Caffeic acid TMS	0.8	Phosphoric acid,O,O,O TMS	3.5***
Citric acid TMS	3.4***	Proline,N,O TMS	0.4
Erythritol TMS	0.9	Pyroglutamic acid,N,O TMS	1.0
Ferulate acid TMS	0.9	Raffinose TMS	1.1
Fructose MeOX1 TMS	1.0	Ribose MeOX2 TMS	4.6
Fructose MeOX2 TMS	1.0	Serine,N,O,O TMS	0.2**
Fumaric acid TMS	1.1	Serine,O,O TMS	0.9
Galactose MeOX1 TMS	0.9	Sorbitol TMS	1.0
Galactose MeOX2 TMS	1.0	Succinic acid TMS	1.1
Gluconic acid TMS	1.0	Sucrose TMS	1.2**
Glucose MeOX1 TMS	1.0	Sucrose-6-phosphate TMS	2.8***
Glucose MeOX2 TMS	0.9	Threonine,N,O TMS	1.1
Glucuronic acid MeOX1 TMS	0.7	Threonine,N,O,O TMS	0.4**
Glutamic acid,N,O,O TMS	0.7	Tyrosine, N,O,O TMS	1.2
Glutamine,N,N,O TMS	0.7	Tyrosine,N,O TMS	1.0
Glyceric acid TMS	1.2	Unknown phosphorylated metabolite TMS	42.4***
Glycerol TMS	1.4**	Unknown phosphorylated metabolite TMS	7.6***
Glycerol-3-phosphate TMS	2.3***	Uracil TMS	0.8
Glycine,N,N,O TMS	0.8**	Urea,N,N TMS	1.0
Histidine, N,O TMS	1.4	Valine,N,O TMS	0.8
Homoproline,O TMS	0.6*	Valine,O TMS	1.3

[a]TMS, trimethylsilyl ester; MeTMS, methyltrimethylsilyl ester; Ee, ethyl ester; MeOX, methoxyamine.
*Means are significantly different at P<0.1.
**Means are significantly different at P<0.05.
***Means are significantly different at P<0.01.

Table 16-5. Differential nonpolar metabolite expression in *lpa3* and wild type maize kernels

Metabolite[a]	*lpa3*:wt	Metabolite[a]	*lpa3*:wt
14:0 Me	1.3**	1HO-22:0 TMS	3.3
15:0 Me	1.5*	2HO-18:0 MeTMS	1.2
16:0 Me	1.2*	2HO-20:0 MeTMS	2.6**
16:0 TMS	1.3**	2HO-22:0 MeTMS	7.2**
16: 1cisΔ7 Me	1.3*	2HO-24:0 MeTMS	3.5**
17:0 Me	1.1	2HO-25:0 MeTMS	2.3**
17: 1cisΔ10 Me	1.3*	25:0	1.1
18:0 Me	1.1	27:0	1.3***
18:0 TMS	1.1	28:0	0.7
18:1cisΔ9 Me	1.1	29:0	1.4*
18:1cisΔ9 TMS	1.3*	30:0	1.0
18:2cisΔ9,12 Me	1.1	31:0	1.4**
18:2cisΔ9,12 TMS	1.5*	33:0	1.2**
18:3cisΔ9,12,15 Me	1.1	ρ-Coumaric acid MeTMS	1.0
20:0 Me	1.4***	β-Sitosterol Me	1.2*
20:1cisΔ11 Me	1.3**	β-Sitosterol TMS	1.7**
20:2cisΔ11,14 Me	1.4	3-Methoxy, 4-hydroxybenzaldehyde TMS	b
21:0 Me	1.1	4-Hydroxybenzene acetic acid MeTMS	1.5
22:0 Me	1.2	4-Hydroxybenzoic acid MeTMS	6.9**
23:0 Me	1.2*	4-Methoxy, 3-hydroxybenzoic acid MeTMS	1.3
24:0 Me	1.4***	4-Methoxy, 3-hydroxycinnamic acid MeTMS	1.0
25:0 Me	1.1	Caffeic acid MeTMS	1.1
26:0 Me	1.4**	Campesterol TMS	1.8**
1HO-12:0 TMS	1.5**	Ferulic acid MeTMS	1.0
1HO-14:0 TMS	1.2	Stigmasterol Me	2.1**
1HO-18:0 TMS	1.2	Stigmasterol TMS	2.1**

[a]Me, methyl ester; TMS, trimethylsilyl ester; MeTMS, methyltrimethylsilyl ester; Ee, ethyl ester.
[b]Not found in wild type.
*Means are significantly different at P<0.1.
**Means are significantly different at P<0.05.
***Means are significantly different at P<0.01.

metabolites in the polar and nonpolar fractions should be interpreted with the presumed effect of the analytical process in mind. We will understand this phenomenon better when we determine the relative amounts of free and bound phenolic acids in maize kernels by LC/MS.

Our targeted and metabolomic analyses revealed several P-containing metabolites that were much more abundant in both *lpa1* and *lpa3* kernels compared to their wild-type controls that developed within the same ear. Several other metabolites were also affected differentially; these are potential targets for possible metabolic rescue of suboptimal yield. The

metabolomic data are not consistent with the presumed phytic acid biosynthetic pathway in either mutant. This is not surprising for less metabolically active mature kernels, thus the need to extend the analyses to embryos isolated from developing kernels. We also plan to analyze *lpa2* kernels in order to investigate the effects of all three low phytic acid mutations on additional P-containing (and other) metabolites measured by GC/MS and LC/MS. LC/MS will allow us to semi-quantify additional metabolites, and determining their empirical formula with high-resolution mass spectrometry will facilitate their identification.

ACKNOWLEDGMENTS

The authors acknowledge the Organizing Committee of the 3rd International Conference on Plant Metabolomics for the inclusion of this paper in an oral symposia and these Proceedings Oliver Fiehn and the Max Planck Institute of Molecular Plant Biology for sharing of their analytical methods and GC/MS libraries, and Jonathan Lightner, Bruce Orman, and Pioneer/DuPont management for their useful discussions and support.

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