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Low Phytic Acid 1 Mutation in Maize Modifies Density, Starch Properties, Cations, and Fiber Contents in the Seed

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ABSTRACT: Monogastric animals are unable to digest phytic acid, so it represents an antinutritional factor and also an environmental problem. One strategy to solve this problem is the utilization of low phytic acid (*lpa*) mutants that accumulate low levels of phytic P and high levels of free phosphate in the seeds; among the *lpa* maize mutants *lpa1* exhibited the highest reduction of phytic acid in the seed. This study indicated that the low phytic acid mutations exerted pleiotropic effects not directly connected to the phytic acid pathway, such as on seed density, content of ions, and the antioxidant compounds present in the kernels. Furthermore some nutritional properties of the flour were altered by the *lpa1* mutations, in particular lignin and protein content, while the starch does not seem to be modified as to the total amount and in the amylose/amylopectin ratio, but alterations were noticed in the structure and size of granules.

KEYWORDS: *maize, phytic acid, starch, ions, storage proteins, lignin content*

INTRODUCTION

The phytic acid, myo-inositol 1,2,3,4,5,6-hexakisphosphate (IP6), stored in seeds represents the form of phosphorus accumulated in plants. The phosphate groups of phytic acid (PA) form salt complexes (phytate) with nutritionally important cations such as calcium, iron, magnesium, potassium, and zinc. During seed ripening PA is deposited in the protein storage vacuole (PSVs) in inclusions called globoids,[1] while in mature maize kernels the PA is mainly localized in the scutellum (80%) and the remainder in the aleurone layer (20%).[2]

The phosphorus stored as PA is made available for the seedling's requirements during germination by the activity of phytases: these enzymes (widespread in nature) catalyze the sequential hydrolysis of phytate.[3]

Monogastric animals are not able to efficiently use phytic acid and the mineral cations bound, due to the absence or low levels of phytase activity in their digestive tracts: the ability of phytate to bind divalent cations (such as Fe^{2+}, Zn^{2+}, and Ca^{2+}) leads to the constitution of insoluble complexes in the digestive apparatus. In this way PA acts as an antinutritional factor, preventing absorption by chelating micronutrients and limiting P bioavailability[4−6] and pepsin activity.[7] These negative properties of PA cause a reduction in the nutritional value of seeds used for human food or animal feed. Furthermore, the presence of undigested P contained in animal excreta can actively contribute to the pollution of surface waters and promote algal blooms (eutrophication).[8] For these reasons, increasing bioavailable phosphorus through reducing the PA in seeds is a target in "biofortification", a process leading to the enhancement of the nutritional value of crops with the aim of countering micronutrient deficiencies (in particular of iron), or "hidden hunger".[9,10]

A genetic approach to tackle this problem can involve genetically modified plants producing seeds with a reduced PA content (presence of exogenous phytase activity in the seeds) and with enhanced iron content and bioavailability due to overexpression of ferritin.[11]

On the other hand, phytic acid, because of its antioxidant properties, might be considered by developed countries' consumers to be an interesting antioxidant compound.[8,10]

Different approaches were taken to solve the nutritional and environmental problems generated by PA in feed and staple foods: for example phytase enzymes can be supplied in postharvest treatments, to increase the available inorganic P. In this way there is a more efficient use of P and a reduction in P in wastes but an increase in both cost and preparation time.

By classical breeding it is possible to create crop germplasm characterized by lower amounts of PA in seeds caused by *low phytic acid* (*lpa*) mutations:[8] the mutants isolated show a loss of function regarding the phytic acid biosynthetic pathway. The *lpa* mutations are classified into three main categories: mutations in the first part of the biosynthetic pathway (from glucose 6-P to myo-inositol(3)monophosphate); mutations in the final step of the PA pathway (from myo-inositol(3)monophosphate

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to PA); and mutations in the storage process of PA in the vacuole.[8,12]

Low phytic acid mutants have been isolated in main crops: in barley,[13−15] in common bean,[16] in pea,[17] in rice,[18,19] in soybean,[20−22] and in wheat.[23] In maize, three *low phytic acid* classes of mutants have been isolated: *lpa1*,[24−26] *lpa2*,[24] and *lpa3*.[27] All of them modify the P repartition without a reduction of total P: in particular *lpa1* attracted researchers' interest for its high ability to reduce PA in grains, resulting in an increase in bioavailable inorganic P.[24−26]

Regarding the *low phytic acid 1* mutant gene, it encodes for ZmMRP4 (GenBank ID: EF586878), a transmembrane transporter of the multidrug resistance-associated protein (MRP) family related to the ATP-binding cassette (ABC) subfamily.[28] The roles of MRP are various and include the transport of several molecules and ions, detoxification, transpiration control, and oxidative stress tolerance.[29−31]

Observations on *lpa* mutants suggested the implication of inositol phosphates, such as phytic acid, in the main plant life processes: this causes negative pleiotropic effects, perturbing the development process and affecting crop yield.[8,26] Major constraints and alterations were identified in abiotic stress tolerance, seed filling, germination rate, and secondary metabolism processes.[15,23,24,32−36]

Studies conducted on P and mineral availability proved the potential contribution of low phytic acid crops to improving the nutritional quality of foods and feed.[37−40]

This work reports on comparative observations among different classes of *lpa1* maize mutants. The analyses were not only related to P content but also displayed differences in physical properties, with potential implications for the practical use of low phytic acid maize flour.

MATERIALS AND METHODS

Genetic Stocks and Sampling Material. The *lpa1-241* and *lpa1-7* mutants were originally isolated from the M2 progeny of chemically (ethyl methane sulfonate) mutagenized populations.[25,26] The *lpa1-1* mutant stock was kindly provided by Dr. Victor Raboy, USDA ARS, Aberdeen, ID, USA.[24] The three alleles were previously introgressed into the inbred line B73. For all genotypes tested in the 2010 field season about 200 plants were sown in adjacent rows, under the same agronomic conditions, in the experimental field of the University of Milan located in Landriano (PV), Italy (N 45°18′, E 9°15′). These plants were selfed and then harvested at the same time at the end of the season. For those containing the nonlethal *lpa1-1* mutation, the ears were shelled and mixed. Three samples of seeds (about 300 seeds each) were milled to obtain the flour used for the following analysis. For the calculation of seed density we used single seeds randomly sampled from the main seed bulk. In the case of *lpa1-7* and *lpa1-241* lethal mutations, we found, using Chen's assay,[41] the +/− ears (assay carried out on flour obtained by pooling a sample of 30 seeds per ear) and selected the single lpa seed (homozygous −/− segregating 3:1 in each +/− ear) again using Chen's assay on aliquots of flour obtained by milling single seeds. By so doing, we selected about 300 seeds that were pooled, milled, and used for P fraction analysis. For the calculation of seed density we used single seeds from selected +/− ears, and after the density measurement the genotypes of each seed were attributed using Chen's assay.

Milling. Flour samples were obtained using a ball mill (Retsch MM200, Retsch GmbH Germany), and seeds were ground for 5 min at 21 oscillations s^{-1} frequency.

Assay for Free Phosphate Content in the Seed. Seeds were ground in a mortar with a steel pestle. One mL of 0.4 M HCl was added to 100 mg of flour obtained. After 1 h at room temperature 100 μL of extract was used for the free phosphate assay, adding 900 μL of Chen's reagent (6 N H_2SO_4, 2.5% ammonium molybdate, 10% ascorbic acid, H_2O [1:1:1:2, v/v/v/v]) in microtiter plates.[41] After incubation of 1 h at room temperature the blue-colored phosphomolybdate complex was observed: the intensity of the blue color is directly proportional to the free phosphate content.

The free phosphate content was quantified by using a spectrophotometer (λ 650 nm) and adopting a series of calibration standards obtained from a stock solution of KH_2PO_4.

Assay for Seed Phytate Content. A modified colorimetric assay was used to quantify phytate levels in maize kernels.[26] A 10 mL amount of 0.65 M HCl was added to 0.5 g of ground kernels in a 15 mL Falcon tube and then incubated in a shaker at room temperature for 16 h overnight. The samples were centrifuged at 2000*g* for 20 min at 10 °C, and the supernatants were transferred into a 15 mL Falcon tube containing 1 g of NaCl. The salt was dissolved by shaking for 20 min at room temperature, and the samples were placed to settle at 4 °C for 1 h or at −20 °C for 20 min. The extract was filtered (using a 0.45 μm nylon syringe filter) and diluted 1:25 in distilled-deionized water.

The calibration standards were prepared from a stock solution of phytic acid dodecasodium salt (Sigma, product no. P-8810, St. Louis, MO, USA).

The colorimetric reaction takes place on adding 500 μL of Wade reagent[42] to 1500 μL of diluted sample and standards in a 2 mL microcentrifuge tube. The tubes were shaken on a vortex and then centrifuged for 10 min at 2000*g*.

The absorbances were measured for both samples and standards using a spectrophotometer (λ 500 nm), and the PA-P content was calculated following the method described in Latta and Eskin.[43]

ICP-MS Analysis. For the ionomic analysis, 10 mL of 65% HNO_3 was added to 100 mg of maize flour samples in Teflon tubes, and the samples were digested by a microwave digestor system (Anton Paar Multiwave 3000, Austria) in Teflon tubes by applying a two-step power ramp (step 1: at 400 W in 5 min maintained for 10 min; step 2: at 1000 W in 10 min, maintained for 15 min).

The mineralized samples were cooled for 20 min at room temperature and then transferred into polypropylene test tubes.

The samples were diluted 1:40 with Milli-Q water, and the elements' concentrations were measured by ICP-MS (Varian 820 ICP-MS, Agilent USA). An aliquot of a 2 mg L^{-1} internal standard solution (^{6}LI, ^{45}Sc, ^{89}Y, and ^{159}Tb) was added to the samples and the calibration curve to obtain a final concentration of 20 μg L^{-1}.

Polyatomic interferences (except for P) were removed by using CRI (collision−reaction interface) with an H_2 flow of 50 mL/min^{-1}.

Seed Density Analysis. In order to measure the density of single kernels, we used a liquid pycnometer of 50 mL volume and deionized water as the displacing liquid.

Starch Granule Size Measurement. In order to measure starch granule size, the endosperm of maize kernels was scraped using a scalpel blade and the flour obtained was transferred directly to a slide with some drops of water for microscopic observation. Images were taken using a Zeiss IMAGE R.D1 microscope equipped with an AxioCam MRc1 digital camera.

Amylose/Amylopectin Measurement. The amylose/amylopectin assays were performed utilizing the commercial kit Megazyme amylose/amylopectin assay (Megazyme Ireland International, Ltd., Bray, Ireland). The procedure was performed according to the manufacturer's recommendations.

DPPH. The DPPH test conducted on maize flour was performed following the procedure described by Doria et al.,[34] based on the procedure proposed by Brand-Williams et al.[44]

Characterization of Proteins by the UTLIEF Method. Analyses were carried out essentially according to ISTA Rules version 2011.[45] The alcohol-soluble fraction of proteins was extracted from individual seeds and separated by isoelectric focusing (IEF) in ultrathin layer gels.

A total of 40 seeds were crushed into fine flour and extracted with a 30% (v/v) solution of 2-chloroethanol for one night, then pelleted with a 20 min centrifugation. The supernatant was transferred into a new microcentrifuge tube, and 15 μL of the extract was loaded in a SERVALYT PRECOTES ultrathin gel, pH 3−10. The focusing was carried out at 2000 V, 6 mA, 12 W and stopped after 2.5 h.

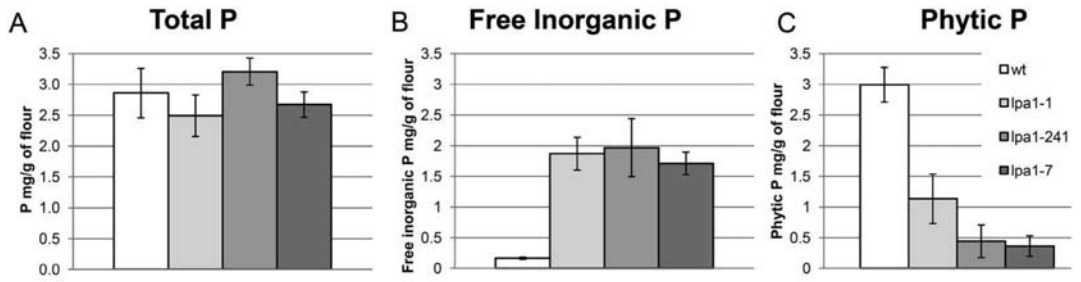

Figure 1. Mature dry kernels were analyzed for free inorganic P (A), total P (B), and phytic acid P (C) quantification. The P was expressed as milligrams of P (atomic weight 31) in 1 g of flour. Confidence intervals at 95% are shown.

After the separation the gel was fixed with a 12% TCA solution for at least 20 min, then washed in a destaining solution (30% EtOH, 5% CH_3COOH) and stained in a 11% CH_3COOH, 18% EtOH, 0.015% Coomassie R250, and 0.045% Coomassie G250 solution for at least 1 h. The gel was washed with destaining solution until the banding pattern was clear and dried overnight at room temperature.

Characterization by Starch Gel Electrophoresis (SGE) of the Isoenzymes. Analyses were carried out according to the CPVO protocol.[46] The isoenzyme extract was obtained from 10 young seedlings (coleoptiles) grown on Petri dishes at 25 °C for four days in the dark. After harvesting the coleoptiles in 1.5 mL microcentrifuge tubes we added 110 μL of 4 °C extraction buffer (16.7% sucrose, 8.3% sodium ascorbate made in deionized water and adjusted to pH 7.4 with L-ascorbic acid). The 1.5 mL tubes with the seedlings and the extraction buffer were then cooled on ice.

The coleoptiles were crushed with a pestle and centrifuged at 4 °C for 15 min at 16 000 rpm; then 25 μL of the extracts was adsorbed on Whatman no. 3 chromatography paper wicks and loaded on two 12.5% starch gels in a histidine-citrate buffer system at pH 5 and pH 6.5, respectively. Appropriate reference maize lines for the recognition of the alleles and a tracking dye for the migration of the samples (50 mg of bromophenol blue dissolved in 100 mL of deionized water) were loaded .

For the recognition of the alleles at the loci ADH (*Adh4*, *Adh6*), PGD (*Pgd1*, *Pgd2*), ACP (*Acp1*), PGI (*Pgi1*), IDH (*Idh1*, *Idh2*), PGM (*Pgm1*, *Pgm2*), and MDH (*Mdhl*, *Mdh2*, and *Mdh4*) we used the SGE at pH 6.5. For the recognition of the alleles at the loci *Mdh3* and *Mdh5*, we used a second electrophoresis histidine-citrate system at pH 5.0.

The electrophoresis was carried out at 4 °C. A constant voltage of 200 V was first applied (maximum current of 150 mA for two 18 × 18 × 1 cm gels was applied for 20 min), and, after the removal of the loading wicks the run continued at a constant voltage of 280 V (maximum current of 180 mA for two 18 × 18 × 1 cm gels), until the bromophenol blue marker migrated 14 cm (4 h).

After electrophoresis the gel is cut horizontally in 1 mm thick slices. Individual gel slices are stained by incubation in the enzyme-specific solutions at 37 °C in the dark.

The evaluation of the results was made by the identification of the alleles at the different loci.

Bromatological Analysis. Chemical analyses of maize samples were performed according to AOAC standard methods.[47] The samples were milled and analyzed for dry matter (DM), ash, crude protein (CP), and ether extract (EE); starch concentration was measured enzymatically. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined using the F57 filter bag system (ANKOM Technology, New York, USA). In particular, NDF was assessed according to the procedure of Mertens,[48] while ADF and ADL were determined according to Van Soest et al.[49] Gross energy was measured using an adiabatic calorimeter (IKA 4000, Staufen, Germany).

Calorimetric Investigations of Mutant Maize Starches. Differential scanning calorimetry (DSC) was performed on flours from dehulled seeds of wild-type and *lpa1-1* mutant maize. A Perkin-Elmer DSC-6 with 60 μL of sealed cells was used. The typical sample overall mass was about 30 mg at either 46% or 75% humidity. The reference cell contained a suitable amount of distilled water. Indium fusion was used for calibration. Measurements were carried out in the 20−150 °C temperature range with a 2.0 °C min^{-1} scanning rate. Heating−cooling−heating cycles were performed. The general trend of the DSC record (excluding transition regions) of the immediate reheating run was chosen as the baseline of the record of the first heating run in order to obtain the trend of the apparent heat capacity, $C_p^{app}(T)$. Once scaled with respect to the baseline, the record reproduces the so-called excess heat capacity, $C_p^{ex}(T)$ (per gram of dry matter), which allowed evaluation of the enthalpy drop ΔH by a straightforward integration.

RESULTS AND DISCUSSION

P Fraction Analysis of *lpa1* Mutants. Quantitative analyses were performed on P fractions contained in the *lpa1-1*, *lpa1-241*, and *lpa1-7* mutant seeds and on wild type seeds as control. No significant alterations in total P amount were observed (Figure 1A): in fact even if the mutations cause approximately a 10-fold increase in the amount of free phosphate (Figure 1B), this was balanced by a reduction of PA (Figure 1C). It was noticed that the *lpa1-241* and *lpa1-7* mutant seeds exhibited the strongest reduction in PA, this seemed to result in a compromised viability of the seeds as reported in previous studies.[25,26]

Ionomic. The mineral and trace element content was determined by inductively coupled plasma mass spectrometry (ICP-MS) analysis. The results showed some differences among the ion compositions of wild type and the *lpa1-1* mutant; in particular Mg and K are accumulated in higher amounts in the mutant (Table 1). No significant differences have been noticed between wt and lpa mutant regarding other single elements; however in comparison with the wild type, the mutant Be, Cd, Cr, Ag, Cu, Zn, Fe, and Co cations were accumulated at lower concentrations, whereas Ni, Hg, As, Na, Al, Mn, and Ca showed higher concentrations. We suggest that ion-homeostasis and phytic acid networks in plant seeds may be linked in some way. This is not surprising when we consider that, as previously reported, PA exhibited a strong chelating effect *in vitro* on mineral cations, especially Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Fe^{3+}, Ca^{2+}, and change in PA content or other indirect pleiotropic effects such as variations in pH value can modify the sequestering ability of PA, leading to different orders of affinity and consequently accumulation in globoids.[1,50]

Previous papers reported different values regarding the mineral nutrients and the trace elements present in *lpa1-1* with respect to the wild-type maize control. Lin and colleagues[51] found statistically significant differences between the *lpa1-1* maize mutation and control in Mg, Ca, Fe, and Mn contents in whole grain: Mg, Fe, and Mn were present in higher amounts in the mutant, while Ca was at a lower level compared to the

Table 1. Mineral Nutrient and the Trace Element Content Determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Analysis Performed on Maize Flour of Wt (Wild Type) and Mutant (*lpa1-1*)[a]

	wt	*lpa1-1*
Ni	0.38 ± 0.04	0.7 ± 0.11
Be	0.03 ± 0.02	nd[b]
Cd	0.02 ± 0.01	nd
Hg	0.08 ± 0.03	0.18 ± 0.07
Co	0.05 ± 0.02	0.03 ± 0.01
Cr	0.06 ± 0.03	0.03 ± 0.01
Ag	0.27 ± 0.14	nd
As	0.05 ± 0.02	0.11 ± 0.02
Na	4.71 ± 1.1	4.93 ± 0.91
Al	5.81 ± 2.4	7.1 ± 2.72
Mn	5.88 ± 0.11	6.18 ± 0.08
Ca	44.40 ± 8.42	60.00 ± 7.21
Fe	25.20 ± 1.20	21.67 ± 0.62
Zn	25.37 ± 0.23	24.51 ± 0.32
Mg	930.56 ± 30	1100.78 ± 35[c]
K	3335.51 ± 65	4133.50 ± 82[c]

[a]The elements were expressed as micrograms per gram of flour. SD is shown. [b]nd, not detected. [c]Different from wt, based on *t* test ($n = 3$, $p < 0.05$).

control. Mendoza and colleagues, in a study on the effect of *lpa1* maize on absorption of iron from tortillas, reported statistically significant differences in unprocessed grain between *lpa1-1* maize and control only for Mg content, which was lower in *lpa1-1* grain.[52] In our work we found significant differences only for Mg and K out of the 16 elements analyzed, but this fact is not surprising if we take into consideration the differences of genetic background of the maize used in these different studies: in the first case a near-isogenic line obtained from the cross A632 × A619,[51] in the second case a flint maize[52] (a variety that is very different, having kernels with a stiff outer layer surrounding the endosperm used to produce tortillas/polenta). In our own case we used the B73 inbred line. Another study on the effect of low-phytate corn and barley on broiler chick performance reported no important differences in either barley or maize with respect to the Ca, Mg, Zn, Cu, and Mn accumulation in whole grains (no statistical analyses were shown for these data) using a dent maize hybrid and the cultivar Harrington for barley.[53]

In the case of rice an increase of calcium, iron, and zinc in the Os-lpa-XQZ-1 mutant was reported, while in the case of Os-lpa-XQZ-1 and Os-lpa-XS110-2 mutants no differences were found.[54] Furthermore the data obtained from studying the accumulation of cations in *lpa* rice mutants in different seasons and locations showed a strong environmental effect.[54] In dicot plants such as soybean, no important modifications in contents of calcium, iron, zinc, and cadmium in mutants (*Gm-lpa-TW75-1* and *Gm-lpa-ZC-2*) have been reported compared to wild type.[54]

It is well known that phenotypic/pleiotropic effects of recessive mutations can be modulated by genetic background, and in the case of *lpa* genotypes we can conjecture that the differences observed in the different studies may be ascribed to this effect as previously discussed. In fact Lorenz and colleagues reported that the P fraction of wild-type maize grains could be modified through breeding and selection, demonstrating that the phytic acid-phosphorus and consequently other mineral nutrients' accumulations are regulated by the interaction among several genes.[55] Another important aspect to consider is the different environments where the plants were cultivated, as reported by Thomas and colleagues in the case of *lpa* rice:[54,56] different mineral concentrations in the soil modify the accumulation of mineral elements in the seeds. In fact we can obtain the biofortification of crops by application of mineral fertilizers and/or by improving the solubilization/mobilization of mineral elements in the soil.[57] Of course further work will be necessary to better understand the effect of the genetic background and of the environment to fully exploit the utilization of these mutants.

Bromatologic and DPPH Analysis. Bromatologic analysis performed on flour obtained from wild-type and *lpa1-1* mutant seeds found quantitative differences in composition with respect to crude protein, lignin content, and ash (Table 2).

Table 2. Bromatologic Analyses Performed on Maize Flour of Wt (Wild Type) and Mutant (*lpa1-1*)[a]

		wt	*lpa1-1*
dry matter	(%)	96.2 ± 0.11	96.2 ± 0.12
crude protein	(%)	9.14 ± 0.41	11.1 ± 0.44[b]
ether extract	(%)	5.56 ± 0.18	5.21 ± 0.16
crude fiber	(%)	2.02 ± 0.27	1.71 ± 0.25
ash	(%)	1.66 ± 0.02	1.93 ± 0.02[b]
starch	(%)	67.2 ± 2.43	69.2 ± 2.13
amylose	(%)	23.0 ± 6.03	22.7 ± 6,26
amylopectin	(%)	44.1 ± 6.02	46.4 ± 6.24
neutral detergent fiber	(%)	13.4 ± 0.39	11.3 ± 0.4[b]
acid detergent fiber	(%)	2.52 ± 0.16	2.09 ± 0.15[b]
acid detergent lignin	(%)	0.49 ± 0.02	0.13 ± 0.04[b]
calorific value	(J/g)	19279 ± 130	18981 ± 186

[a]Confidence intervals at 95% are shown. [b]Different from wt based on *t*-test ($n = 3$, $p < 0.05$).

In the work of Mendoza and colleagues[52] on the effect of the *lpa1* mutation of maize on absorption of iron from tortillas and in the work of Li et al.[58] no differences were reported for crude protein and ash. Furthermore in Li's work, in agreement with our results, a lower content in fiber was reported in the mutant.[58] Another paper also reported no differences for ash but a higher level of crude protein in the *lpa1-1* mutant with respect to control as in our case.[53] However as discussed previously for the cation content, the different genetic backgrounds can explain these different results.[52] The higher content of ash observed in the *lpa1-1* mutant reported in the last-mentioned work[52] is in agreement with the higher levels of Mg and K detected in the mutant, while for the crude protein level this result was also observed in the work of Jang and colleagues using a hybrid homozygous for the *low phytic acid 1-1* allele.[53] An important point is that our data regarding fiber and lignin content in the *lpa1-1* mutation in maize are in agreement with the results obtained by studying the *low phytic acid* mutation in barley that perturbs the expression of genes involved in the cellular biosynthetic pathways, causing a reduction in acid detergent fibers and in lignin content.[59]

On the other hand, a different study on *lpa* mutations in barley reported in all the cases a higher content of ash and crude protein in whole grain, as found in maize in our work.[60]

The alterations in fiber structure, originated by phytic acid pathway modification, resulting in an improvement in hydrolyzability, could enhance bioethanol production from cellulosic

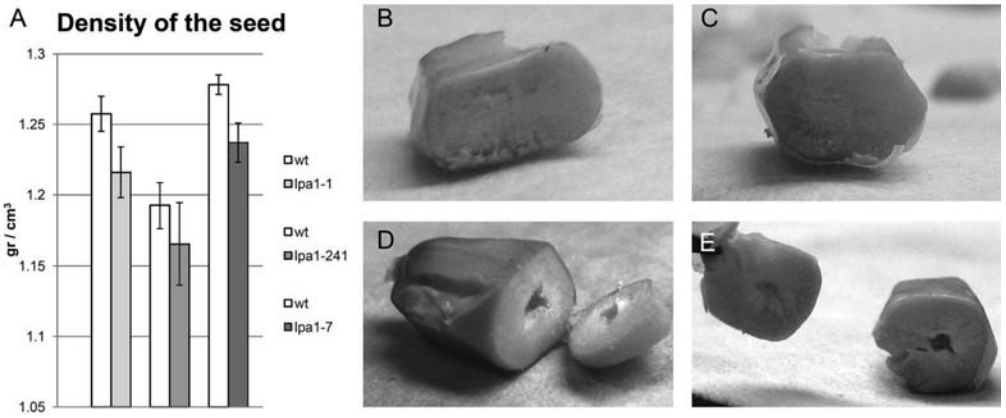

Figure 2. (A) Specific weight of wild-type and mutant kernels; density expressed as grams per cubic centimeter. Confidence intervals at 95% are shown. Transversal section of wild-type (B and C) and *lpa1-7* kernels (D and E). The cavity in the endosperm is clearly visible in pictures D and E.

material[61,62] and thus can be seen as a further biotechnological application of these mutants.

Alterations were also noticed in physical properties, in particular a difference in consistence and texture of flour after the milling process: the flour obtained from the *lpa1-1* mutant is less vitreous and sandy than that from the wild type (data not shown). A difference in density was also observed between wild-type and *lpa1-7* mutant seeds (Figure 2A): the transverse section of wild-type and mutant mature kernels revealed the presence of an unexpected small cavity in the endosperm area of the mutant (Figure 2B–E). These traits can contribute to the previously reported reduction in yield of the low phytic acid mutant.[24,33] It is possible to argue that the phytic acid role in metabolism could be related to the seed filling process during maturation, contributing to pleiotropic effects characteristic of this class of mutants.[26,33] The side effects of phytic acid reduction were also reported in other aspects of plant structural properties. The reduction in phytic acid content can lead to a release of divalent cations that form an insoluble complex with pectin. This induces a firmness of texture causing hardening of the seeds and leads to increases in cooking time, as shown in pinto bean.[63]

Measurement of ARP (antiradical power) by means of the DPPH test supported the data obtained by Doria et al.[34] on *lpa1-1* seeds, confirming that they contain more ARP (about 1.5-fold) than wild-type seeds (data not shown). The reduction of phytic acid, an antioxidant, does not compromise the antiradical properties of low phytic acid flour. This may suggest that the increased oxidative stress in *lpa* seeds, due to the lower level of phytic acid compared to wild type during maturation, can contribute to stimulate the production and accumulation of larger amounts of other antioxidant compounds. However considering previous results obtained by artificially accelerating aging and analysis of the levels of protein carbonylation and apurinic/apyrimidinic sites in DNA, we can state that *lpa1* mutations suffer from oxidative stress but have more ARP.[34]

Proteins and Isoenzymes Characterization. The electrophoretic characterization of the *lpa1* mutant line showed, in comparison with the wild type, some differences along the whole pH range investigated. In particular in a pH range between 9.5 and 10.7 the zein pattern in mutant seeds displayed a detectable clear band, absent in wild-type seeds. The pattern of the *lpa1* line showed a band in the highest pH region in all 40 seeds examined, while this band was absent in all the wild-type extracts (Figure 3A).

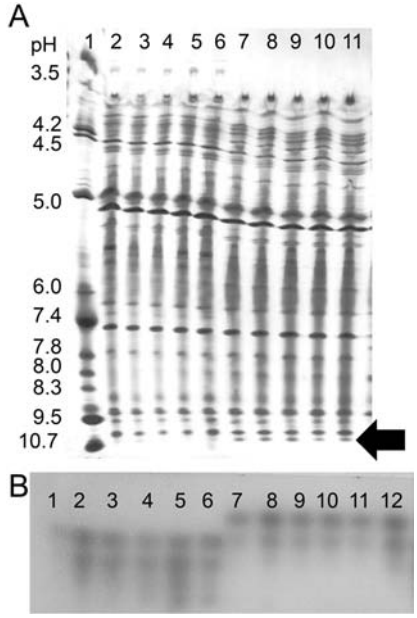

Figure 3. (A) UTLIEF (ultrathin-layer isoelectric focusing) total protein (zeins) pattern. Lane 1: marker; lanes 2–6: wild type; lanes 7–11: *lpa1-1*. *lpa1-1* seeds showed some differences in the basic region (pH 9.5–10.7). (B) PGI zymogram. Lane 1: line F7 Pgi 4/4 allele; lanes 2–6: wt; lane 7–11: *lpa1-1*; lane 12: line A632 Pgi1 5/5 allele. Wt showed the Pgi1 4/4 allele, while the lpa1/lpa1 presented the 5/5 allele.

A further characterization based on the genetic description of seven enzymatic systems present in the mutant and wild-type coleoptiles was performed. In the *Pgi1* locus we observed a different pattern between mutant and wild type. The mutant shows the allele *Pgi1* 5/5, while the wild type contains the most frequent allele, 4/4 (Figure 3B).

The difference between the two lines could be due to the origin of the material provided: *Pgi1* and *lpa1* are both on chromosome 1 (bin 1.02), so the selection of the mutant *lpa1* may drag the 5/5 allele from the original "Early ACR" parent inbred line used for the isolation of the first *lpa1*.[24] Furthermore, *Pgi1* pattern 5/5 is typical of the A632 line (data not shown), used as pollen donor parent for the mutagenesis treatments performed in the "Early ACR" to obtain the *lpa1* mutant.

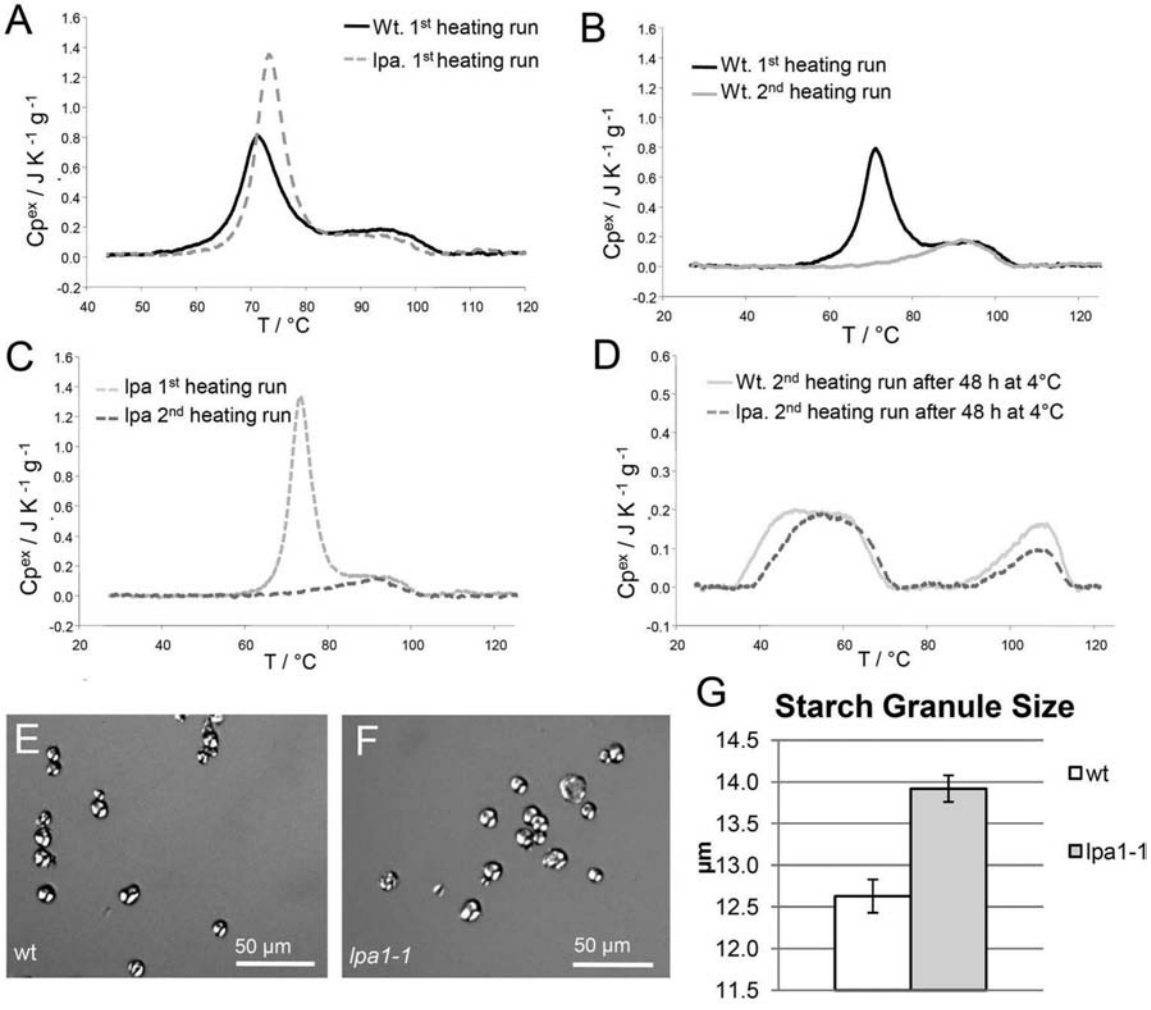

Figure 4. (A–C) DSC records of the first and second heating run (2 °C/min) of maize flour samples (75% humidity): (A) comparison of wild-type and lpa1 maize flours, first heating run; DSC records of the first and second heating run for wild type (B) and *low phytic acid 1-1* (C) show the signals related to the starch gelatinization (first peak) and amylose–lipid dissociation (second peak). DSC records of the second heating run (after 48 h storage at 4 °C) of maize flour samples (46% humidity) for wild type and *low phytic acid 1-1* (D): amylopectin retrogradation (first peak) and amylose–lipid dissociation (second peak) are observed. Starch granules from wild-type (E) and mutant kernels (F). Starch granule size comparison among wt and low phytic acid. Confidence interval at 95% is shown (G).

Both these observations suggested that the wild-type and *lpa1-1* line are distinguishable not only on a genetic basis but also by the different composition of zeins. For a deeper characterization of the qualitative zeins pattern, the pH range investigated could be split in the two extreme pH regions (pH 3 to 6 and pH 7 to 10).

Starch Analysis. As shown in Table 2 no differences were found in total starch content and amylose/amylopectin ratio, as reported previously in the work of Tadmor and colleagues in 2001.[64] However in this work we also investigated differences in starch properties, namely, the rate and extent of starch gelatinization and retrogradation, as well as the formation of amylose–lipid complexes, which were searched for by means of DSC investigations. An interesting picture was offered by the traces of the excess heat capacity, C_p^{ex}, of the wild-type and mutant variety (Figure 4A). Each trace shows a shouldered peak, the main component of which is related to the fusion of the amylopectin crystal phases within the starch granule, while the shoulder corresponds to the fusion of the amylose–lipid complexes.

Since the former process is fully irreversible, while the latter is partially reversible on cooling, the DSC collected in a second heating run, performed just after cooling, shows only the signal relevant to the fusion of the amylose–lipid complexes (Figure 4B,C). If the gelatinized starch samples are left to rest at 4 °C for a couple of days, the amylopectin gel undergoes a partial recrystallization that is commonly dubbed "retrogradation". The DSC heating run of a retrograded sample shows a trace with a broad endothermic peak (that corresponds to the fusion of these crystals) followed by another peak related to the fusion of the amylose–lipid complexes (Figure 4D). The former broad signal reveals the presence of various crystal types: the larger the signal, the larger the dispersion of crystal patterns.

Looking at the traces reported in Figure 4A–D, the differences between wild and mutant maize starches are definitely of minor relevance and mainly concern amylopectin. As for the amylopectin fusion, the enthalpy drop is practically the same (12 ± 1 J/g) for either type, although the onset temperature is a little lower for the wild type. The comparison of the DSC profiles (Figure 4A) may indicate a minor

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dispersion (sharper peak) and a more compact structure of the starch granules of the mutant maize, or a smaller extension of surface defects (pores and crevices), which can also justify a delay in the granule hydration and swelling. This would be in line with the microscopy evidence (Figure 4E,F) that the wild-type maize has smaller starch granules (Figure 4G). The granule size of starch can be of great interest for industrial applications where starch is used as a filler, such as in the paper industry:[65] for example, big starch granules give higher swelling power and viscosity.[66]

The amylopectin of the starch from the mutant variety undergoes retrogradation to a smaller extent and shows a smaller dispersion of crystal patterns (Figure 4D). The signal relevant to the fusion of the amylose−lipid complexes reveals a lower enthalpy for the mutant type: this may be due to a lower lipid content or to a different structure of the amylose. Both amylopectin retrogradation and formation of amylose−lipid complexes are certainly mediated by the surrounding nonstarch medium, but the present data do not allow any definite interpretation. Data coming from the measurement of amylose/amylopectin ratio and content reveal no difference in amylose percentage: 34.3% in the wild type and 32.8% in *lpa1-1* of total starch (Table 1). These results thus suggest that differences observed are not to be attributed to modifications of the starch composition.

At either 75% or 46% humidity samples (some data are not shown) allow the same conclusions as regards the starch component. Nonetheless, the lower content of phytic acid in the mutant variety may imply differences in the behavior of water, so as to affect the overall behavior of the final product based on maize flour. For instance, since changes of ionic concentration are present in the mutant variety, it is possible to expect a different RH of the related flour, at a given water content. These aspects are the subject of further investigations, now in progress, that will constitute a more detailed study (e.g., attitude to forming "resistant starch", Knudsen TG investigations[67] to evaluate RH of water in dough samples prepared from mutant maize flours, etc.) and the use of complementary techniques to explain differences in some properties of the dough.

Further studies could evaluate whether the *lpa* mutations can perturb phosphorus homeostasis, producing effects on starch phosphorylation.[68,69]

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Notes

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