Phytic acid (PA), myo-inositol-1,2,3,4,5,6-hexakisphosphate, is the main storage form of phosphorus in plants. It is localized in seeds, deposited as mixed salts of mineral cations in protein storage vacuoles; during germination, it is hydrolyzed by phytases. When seeds are used as food/feed, PA and the bound cations are poorly bioavailable for human and monogastric livestock due to their lack of phytase activity. Reducing the amount of PA is one strategy to solve these problems and is an objective of genetic improvement for improving the nutritional properties of major crops. In this work, we present data on the isolation of a new maize (Zea mays L.) low phytic acid 1 (lpa1) mutant allele obtained by chemical mutagenesis. This mutant, named lpa1-7, is able to accumulate less phytic phosphorus and a higher level of free inorganic phosphate in the seeds compared with wild type. It exhibits a monogenic recessive inheritance and lethality as homozygous. We demonstrate that in vitro cultivation can overcome lethality allowing the growth of adult plants, and we report data regarding embryo and leaf abnormalities and other defects caused by negative pleiotropic effects of this mutation.

**Key words:** ATP-binding cassette (ABC) transporter, inositol phosphates, low phytic acid, maize, multidrug resistance-associated protein (MRP), mutagenesis

Phytic acid (PA) (myo-inositol-1,2,3,4,5,6-hexakisphosphate; InsP6) is ubiquitous in eukaryotic cells and constitutes the major storage form of phosphate in plant seeds (from 60% to 80%). During maturation, it is accumulated in the protein storage vacuole in inclusions called globoids; the phosphate groups present in PA are able to form phytate salts (phytin) binding important mineral cations such as calcium, magnesium, potassium, iron, and zinc (Lott et al. 2000). In mature maize kernels, 80% of PA is localized in the scutellum and the remaining 20% in the aleurone layer (O’Dell et al. 1972). The phosphorus stored as PA is remobilized during germination by phytase enzymes: these are also found in many microorganisms (Labouré et al. 1993).

Regarding the involvement of P in agricultural production and its sustainability, it has been estimated that nearly 50% of elemental P used yearly in global agricultural activities is accumulated in the PA (Lott et al. 2000).

PA forms mixed salts with mineral cations that are excreted by monogastric animals and humans because they do not have phytase activity in their digestive systems. Considering that seeds are an important component of animal feed and human food, the limitations of phosphorus and micronutrients bioavailability imply a decrease in their nutritional value. Furthermore, the undigestible phosphorous contained in excreted phytin can contribute to water pollution (eutrophication) (Raboy 2009).

These negative effects have led to breeding programs that have the aim of reducing the PA content in the seeds of several cultivated plants. The main way to reach this result by conventional breeding is the isolation of low phytic acid (lpa) mutations, capable of restraining the biosynthesis or the storage of PA in the seed; the increased P and mineral cation bioavailability in lpa seeds is confirmed by nutritional trials (Mendoza et al. 1998; Hambidge et al. 2004, 2005).

The lpa mutations can be classified into 3 categories: mutations affecting the first steps of the biosynthetic pathway (from glucose 6-P to myo-inositol[3]-monophosphate); mutations perturbing the end of the PA pathway (from myo-inositol[3]-monophosphate to PA), and mutations affecting the transport of PA to the vacuole (Raboy 2009; Panzeri et al. 2011) (Supplementary Figure 1).

In several crops, lpa mutants have been isolated by distinct methods: in barley by chemical mutagenesis (Larson et al. 1998; Rasmussen and Hatzack 1998; Bregitzer and Raboy 2006), in soybean by chemical and physical mutagenesis (Wilcox et al. 2000; Hitz et al. 2002; Yuan et al. 2007), in wheat by chemical mutagenesis (Gutierrez et al. 2004), in common bean by chemical mutagenesis (Campion et al. 2009), and in rice by physical and chemical mutagenesis (Larson et al. 2000; Liu et al. 2007).

In maize, 3 lpa mutants have been isolated: lpa1 (Raboy et al. 2000; Pilu et al. 2003) and lpa2 (Raboy et al. 2000) by chemical mutagenesis and lpa3 by transposon tagging (Shi et al. 2005) (Supplementary Table 1).

Compared with the other mutations in maize, lpa1 exhibited the major reduction of PA in the seed, this comes with a proportional increase of free P without changing...
the total P content. Taking advantage of this property, lpa mutants can be recognized by the HIP (high inorganic phosphate) phenotype of the seeds (Raboy et al. 2000; Pilu et al. 2003). The Lpa1 gene encodes for ZmMRP4 (accession number EF586878), a multidrug resistance-associated protein (MRP) belonging to the subfamily of ATP-binding cassette (ABC) transmembrane transporters (Shi et al. 2007). MRP proteins are implicated in different roles like the transport of organic ions and anthocyanins, detoxification of xenobiotic compounds, transpiration control, and tolerance to oxidative stress (Swarbreck et al. 2003; Goodman et al. 2004; Klein et al. 2006). The role of this MRP protein is not completely understood, but it is fundamental for PA accumulation and viability of seeds. lpa mutants isolated in rice (Xu et al. 2009) and soybean (Wilcox et al. 2000; Saghai Maroof et al. 2009) are related to defects in homologues of the maize ABC transporter.

It was observed that lpa mutations found in several crops usually bring pleiotropic effects on plant and seed performance, such as reduced germination and emergence rate, lower seed filling, weakening in stress resistance, and alteration in the accumulation of anthocyanin (Raboy et al. 2000; Meis et al. 2003; Pilu et al. 2005; Bregitzer and Raboy 2006; Guttieri et al. 2004; Doria et al. 2009; Cerino Badone et al. 2010; Maupin et al. 2011).

The presence of pleiotropic effects shows that lpa mutations influence not only the seed but also the whole plant and its production. This can reflect the relevance of inositol phosphates as multifunctioning molecules and their involvement in fundamental signaling and developmental pathways, like DNA repair, RNA editing, chromatin remodeling, and control of gene expression (Raboy 2009).

Data from previous studies showed that mutations arise at high frequency at the maize lpa locus and indicated the involvement of an epigenetic event of paramutation in the genetics of this trait (Pilu et al. 2009; Raboy 2009; Pilu 2011).

In this work, we report data regarding the isolation and the characterization, under different aspects, of a new lpa mutant in maize, allelic to lpa1. This mutation is monogenic, recessive, and lethal in the homozygous state. We also present the results of physiological analysis, histological observations, and considerations regarding the effects of the lpa1 mutations on the plant.

Materials and Methods

Mutant Isolation and Genetic Analysis

Starting from the K6 inbred line, we generated a population of EMS (ethyl methanesulfonate)-induced mutants (about 300 M2 ears) using the seed treatment method (Neuffer et al. 1997a). We screened this population by the molybdate staining assay that was able to recognize the HIP phenotype (see Assay for Free Phosphate Content in the Seed). The newly isolated mutant was crossed with the 2 lpa1 mutants (lpa1-1 and lpa1-241) in all pairwise combinations in order to assay their complementation pattern.

Assay for Free Phosphate Content in the Seed

We ground seeds in a mortar with a steel pestle, and 100 mg of flour obtained was extracted for 1 h at room temperature adding 1 ml 0.4 M HCl. One hundred microliter of extract were used for the free phosphate assay adding 900 μl of Chen’s reagent (6 N H2SO4:2.5% ammonium molybdate:10% ascorbic acid:H2O [1:1:1:2, v/v/v/v]) in microtiter plates (Chen et al. 1956). After incubation of 1 h at room temperature, we observed the blue colored phosphomolybdate complex whose color intensity is proportional to the free phosphate content.

We evaluated the presence of the HIP phenotype either by visual inspection or by quantifying the free phosphate content using a spectrophotometer (λ, 650 nm) and adopting a series of calibration standards prepared from a stock solution of KH2PO4.

In order to preserve the growth capacity of the seed, we performed a nondestructive assay for the HIP phenotype. We obtained a small amount of scutellum flour from a single incision using an electric drill. Subsequently, the extraction was performed in microtiter for 1 h at room temperature using 200 μl of 0.4 M HCl, then we added 800 μl of Chen’s reagent. After 1 h, we recognized the HIP phenotype by visual inspection.

Assay for Seed Phytate Content

We modified the colorimetric assays of Gao et al. (2007) to quantify phytate levels in maize kernels. We added 10 ml of 0.65 M HCl 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. We centrifuged at 2500 rpm for 20 min at 10 °C, and we transferred the supernatant in a 15 ml Falcon tube containing 1 g of NaCl. We dissolved the salt by shaking for 20 min at room temperature, then we placed the sample to settle at 4 °C for 1 h or at −20 °C for 20 min. After that, the extract was filtered (using a 0.45 μm nylon syringe filter) and diluted 1:25 in distilled–deionized water.

We used a series of calibration standards prepared from a stock solution of PA dodecasodium salt (product no P-8810; Sigma, St Louis, MO).

We started the colorimetric reaction adding 500 μl of Wade reagent (Gao et al. 2007) to 1500 μl of the diluted sample and standards in a 2 ml eppendorf tube. We mixed on a vortex and then centrifuged the tubes for 10 min at 2500 rpm.

We measured the absorbance of the color reaction products for both samples and standards using a spectrophotometer (λ, 500 nm), and in order to calculate of the PA-P content, we followed the method described in Latta and Eskin (1980).
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).

After 20 min of cooling time, we transferred the mineralized samples into Polypropylene test tubes.

We diluted the samples 1:40 with MILLI-Q water, and we measured the concentration of P by ICP-MS (Varian 820 ICP-MS) as $^{31}$P. We added an aliquot of a 2 mg l$^{-1}$ of an internal standard solution ($^{61}$Li, $^{45}$Sc, and $^{89}$Y) to both samples and calibration curve to give a final concentration of 20 $\mu$g l$^{-1}$.

Embryo Rescue

Mature dry seeds were sterilized with 5% (v/v) sodium hypochlorite for 15 min, then incubated in sterile distilled water in rotating flasks at 30 °C for 18 h. We removed embryos aseptically and transferred them to Murashige and Skoog salt mixture (pH 5.6; product no M5519, Sigma) containing 2% (w/v) sucrose, solidified with 0.8% (w/v) agar (Phytagar, product no P8169; Sigma).

We incubated the cultures in a growth chamber at 25 °C with a 14/10 light/dark photoperiod. The light source consisted of 4 cool white (F36T12/CW/HO) fluorescent lamps from GTE SYLVANIA (Lighting Products Group, Danvers, MA). The distance between light sources and seeds was 50 cm. The light intensity was 0.785 $\mu$mol m$^{-2}$ s$^{-1}$.

5-Aza-2’-Deoxyctydine (Azacytidine) Treatment

We sterilized mature dry seeds with 5% (v/v) sodium hypochlorite for 15 min, then incubated the seeds in 20 ml of 30 $\mu$M 5-aza-2’-deoxyctydine (product no A3656; Sigma) in rotating flasks at 30 °C for 18 h. We incubated control seeds in 20 ml of deionized water under the same conditions. After that, we performed the embryo rescue on the treated/control seeds as previously described.

Plant Growth Regulator Treatments

We removed embryos aseptically as previously described and transferred them onto the Murashige and Skoog salt mixture medium (pH 5.6; product no. M5519; Sigma) containing 2% (w/v) sucrose, solidified with 0.8% (w/v) agar (Phytagar, product no P8169; Sigma). We supplemented the medium with the hormones IAA or GA, each at a concentration of 10 $\mu$M. We incubated the cultures in a growth chamber at 25 °C with a 14/10 light/dark photoperiod.

Histological Analysis

We performed light microscopy observations on mature seeds. We soaked in water for 18 h wild-type and mutant seeds, after that we fixed the seeds for 24 h in freshly prepared 3:1 100% ethanol/glacial acetic acid at 4 °C. We placed the fixed material in 70% (v/v) ethanol and stored at 4 °C until processed. After dehydration in an ethanol series and embedding in Paraplast Plus (Ted Pella, Inc. and Pelco International, Redding, CA), we cut sections at 15 $\mu$m, serially arranged, and stained with safranine-fast green as described by Ruzin (1999).

Leaf Trichomes Measurements

We measured the length and the density of leaf trichomes on images of the leaves’ margins obtained using a stereo-scpe equipped with a CCD camera.

Determination of Chlorophyll and Carotenoids

We performed the analysis on mature apical leaves collected at the flowering stage. We followed the method and formulae in the paper of Arnon (1949) regarding the extraction and the quantification of the amount of chlorophyll (a, b, and a+b) and carotenoids.

Lpa1 Allele Molecular Genotyping

We performed a molecular analysis using ZmMRP4 sequence-specific amplification polymorphism (S-SAP) markers able to distinguish the presence of $\text{lpa1-1}$ allele in $\text{lpa}$ mutants.

The allele-specific forward primers were designed on a single nucleotide substitution polymorphism in the $\text{ZmMRP4}$ 10th exon (Shi et al. 2007) (Supplementary Figure 2).

The $\text{Lpa1}$ wild type–specific forward primer was ZmMRP430L (5’-GTACTCGATGAGGCGACACGC-3’), whereas $\text{lpa1-1}$ mutation-specific forward primer was ZmMRP432L (5’-GTACTCGATGAGGCGACAGTG-3’).

The reaction mixture of the wild-type $\text{Lpa1}$ allele-specific amplifications contained an aliquot of genomic DNA, 1× Green Go Taq buffer (Promega, Madison, WI), 2.5 mM MgCl$_2$, 0.2 $\mu$M each of dATP, dCTP, dGTP, and dTTP, 0.3 $\mu$M of forward ZmMRP430L-specific primer, 0.3 $\mu$M of reverse ZmMRP410R primer (5’-CCTCTCTATATA-CAGCTCGAC-3’), and 1.25 unit of Go Taq Flexy DNA polymerase (Promega), in a final volume of 25 $\mu$L. The reaction mix underwent an initial denaturation step at 94 °C for 2.5 min, 37 cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 1 min, extension at 72 °C for 1.5 min. Extension at 72 °C for 5 min was performed to complete the reaction. The wild-type $\text{Lpa1}$ allele-specific amplification product was 468 bp long.

The reaction mix of the $\text{lpa1-1}$ allele-specific amplifications was identical to that of wild-type $\text{Lpa1}$ allele-specific amplifications, except that 0.3 $\mu$M of ZmMRP432L $\text{lpa1-1}$-specific primers were used instead of ZmMRP430L. The reaction mix underwent an initial denaturation step at 94 °C for 2.5 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 1 min, extension at 72 °C for 1.5 min. Extension at 72 °C for 5 min was performed to complete the reaction. The mutant $\text{lpa1-1}$-specific amplificate was 468 bp long.

We loaded amplification products on 1% (w/v) agarose gels and visualized them by ethidium bromide staining under ultraviolet light.
Lpa1 Expression Analysis

We extracted total RNA from frozen leaves sampled from wild-type and lpa1-7/lpa1-7 plants using the method described by van Tunen et al. (1988). We used reverse transcriptase (RT)-PCR to detect ZmMRP4 gene transcripts. The first strand cDNA was synthesized using an oligo (dT) primer from total RNA. We performed on all RNA samples a DNase (1U µg⁻¹) treatment before cDNA synthesis. The first strand cDNA was used as template for PCR, amplification reactions contained an aliquot of cDNA, 1× Green Go Taq buffer (Promega), 2.5 µM MgCl₂, 0.2 µM each of dATP, dCTP, dGTP, and dTTP, 0.3 µM of each primer, and 2.5U of Go Taq Flexy DNA polymerase (Promega), the final volume being 25 µl. The reaction mix underwent 34 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 5 min in order to complete the reaction. The concentration of the different samples was standardized using specific primers for the orange pericarp 1 (orp1) gene, which encodes the β-subunit of tryptophan synthase (Wright et al. 1992). We amplified orp1 sequences using the following primers: the forward primer, 5’-AAGGACGTGCACACCGC-3’ and reverse primer, 5’-CAGATACAGAACAACAACTC-3’. The length of the amplified product was 207 bp. By successive dilutions of cDNA, we obtained similar orp1 amplification signals in the different samples.

In order to detect ZmMRP4 expression, we used specific primers designed on ZmMRP4 exon sequence (Shi et al. 2007): ZmMRP4+5135F (forward primer 5’-TCATGGTGTAAGTTGTATGTTTC-3’) and ZmMRP4+6206R (reverse primer 5’-CCTCTCTATATACAGCTCGAC-3’) as described by Pilu et al. (2009).

We observed a 677 bp amplification product after 33 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. We loaded the amplification products on 2% (w/v) agarose gels and visualized them by ethidium bromide staining under ultraviolet light.

Results

Mutant Isolation, Inheritance, and P Phenotypic Features

With the aim to isolate new maize lpa mutants, we performed a seed mutagenesis treatment with EMS (Neuffer et al. 1997a). Because wild-type mature maize seeds contain high amount of phytic phosphate and low free phosphate content, we screened the mutagenized population (ca. 300 M² families) looking for seeds containing high levels of free phosphate (HIP phenotype), a typical feature of lpa mutants (Rasmussen and Hatzek 1998; Raboy et al. 2000; Pilu et al. 2003).

We isolated one putative mutation that we named lpa1-7 after the allelism test with 2 lpa1 mutants (lpa1-1 and lpa1-241) isolated to date (data not shown). To confirm this finding, we followed the segregation of the progeny obtained from the cross lpa1-7/+ X lpa1-1/lpa1-1 using either the HIP phenotype or the molecular genotyping using ZmMRP4 S-SAP markers able to distinguish the lpa1-1 mutation from other Lpa1 alleles. The results coming from the S-SAP molecular marker confirmed that lpa1-7 was a new lpa1 allele (Supplementary Figure 2). The lpa1-7/lpa1-7 homozygous plants did not appear through several cycles of self-pollination; furthermore, we observed that lpa1-7 homozygous HIP seeds (obtained by conservative assay) were unable to germinate under field conditions and in filter paper germination tests (Supplementary Figure 3). Taken together, these results were compatible with a monogenic recessive behavior of lpa1-7 responsible for lethality in the homozygous state caused probably by the strength of this mutation, in fact it causes approximately a 10-fold increase in the amount of free phosphate (Figure 1A) and a reduction of about 80% of PA compared with the wild-type control (Figure 1B), without a significant alteration in the total P amount (Figure 1C).

It is known that Lpa1 locus undergoes spontaneous silencing with high frequency generating epialleles such as Figure 1. Mature dry seeds were assayed for free inorganic P (A), phytic acid P (B), and total P (C). The P fractions were expressed as milligram of P (atomic weight = 31) on gram of flour. Confidence intervals at 95% are shown.
lpa1-241 (Pilu et al. 2009; Pilu 2011) and other lpa1 alleles isolated in the past (Raboy V, personal communication) with an anomalous inheritance for a recessive trait. With the aim of establishing the origin of our new mutant, we performed RT-PCR analysis with the aim to evaluate the effect of the mutation on the expression of Lpa1 gene encoding for ZmMRP4, the transmembrane transporter of In6P in the vacuoles. We conducted the experiment on leaves of wild-type and homozygous lpa1-7 plants obtained by the same embryo rescue treatment. The expression of ZmMRP4 gene did not show significant variation between the mutant and the wild type (Supplementary Figure 4).

Furthermore, we performed 5-Aza-2’-Deoxycytidine (Azacytidine) treatment on lpa1-7 mutants in order to evaluate its effect on the negative pleiotropic effects caused by mutation. In fact, previous studies showed that lpa1-241 seed treatment with this demethylating agent was able to restore partially the pleiotropic effects compared with untreated controls (Piluet al. 2009). Neither treated wild-type seeds nor homozygous lpa1-7 seeds revealed significant differences compared with untreated controls (data not shown).

Figure 2. Negative pleiotropic effects of lpa1-7 on seedling growth in vitro (A) and on plants (B). The mutation causes a defective primary root in seedling (C). Longitudinal sections of mature wild-type (D) and lpa1-7 (E) kernels. Magnification of wild-type (F) and lpa1-7 (G) root primordia. The sections were stained with safranine-fast green (gm, ground meristem; sc, scutellum; sh, shoot; rt, root; pr, procambium). Bar: 500 μm. Leaf area of mature wild-type (H) and lpa1-7 (I) plants. Magnification of wild-type (L) and lpa1-7 (M) leaf margin and trichomes. Bar: 100 μm.

Embryo-Rescue and Histological Analysis of lpa1-7 Embryo

In order to overcome the lpa1-7 homozygous failure in germination, we performed embryo rescue on mutant seeds and wild-type siblings as control.

Embryo cultures on MS medium of lpa1-7/lpa1-7 mutants could restore lacking germination. The wild-type siblings germinated and grew regularly, whereas only a fraction of mutants generated seedlings: they were characterized by slow growth and abnormal morphological particular at the level of the root apparatus (Figure 2A,B). We observed that all mutants failed to produce a functional primary root; the root stopped its elongation at an early stage, but the development of secondary roots partially compensated for this lack (Figure 2C). Results from the embryo rescue experiment on MS medium with added IAA or GA hormones were not significantly different from those recorded using the standard medium (data not shown).

In order to investigate the alterations observed in embryo rescue experiments, we performed histological analysis on longitudinal sections of lpa1-7/lpa1-7 and wild-type kernels. We observed a reduced dimension of mutant embryos (Figure 2E) compared with the wild type (Figure 2D), we also noticed that the lpa1-7 mutation can alter the structures of the embryo. The mutant root primordium appeared not properly aligned with the embryo body (Figure 2E); furthermore, the area between the root apical meristem and the embryo shoot displayed less organized cellular structures. We observed this defect in the ground meristem and in the procambium (Figure 2F,G).

Characterization of Lpa1-7 Homozygous Rescued Plants

We transplanted the mutant seedlings obtained by embryo rescue and corresponding wild siblings in pots placed in a greenhouse (Figure 2B). We noticed a slow growth rate of the mutants during the whole life cycle, however, we obtained adult fertile plants, and after selfing, each plant produced one small ear with a few seeds having all HIP phenotype as expected for Lpa1-7/Lpa1-7 plants (data not shown).

Observing carefully the leaves of homozygous lpa1-7 plants, we noted pale green stripes between the leaves’ venation (Figure 2H,I). This observation was confirmed by analyzing the mature leaves of mutant plants that displayed a significant reduction in the amount of chlorophyll (a, b, and a + b) and carotenoids (Figure 3C). Furthermore, analysis of the margin of mutant leaves with a stereoscope equipped with a CCD camera showed a significant decrease in the trichomes’ length, accompanied by an increase in leaf trichomes’ density (Figure 2M) compared with wild siblings.
In this work, we present data regarding an lpa mutant obtained by chemical mutagenesis, we isolated a recessive lpa maize mutant, named provisionally lpa-1, obtained by chemical mutagenesis, we isolated a recessive lpa mutant, named provisionally lpa-1, renamed the new lpa allele as lpa-1. Because it is known that maize lpa alleles can be affected by epigenetic events at a relatively high rate (Pilu et al. 2009; Raboy 2009; Pilu 2011), we conducted 2 experiments to ascertain the nature of lpa-1 mutation (gene silencing vs. sequence mutation), we checked the ZmMRP4 gene expression, and we performed a 5-Azacytidine (a demethylating agent) treatment of the seeds. The gene expression analysis of ZmMRP4 conducted on lpa-1 mutant did not reveal significant variations between the mutant and the wild type (Supplementary Figure 4), and the 5-Azacytidine treatment did not show differences compared with untreated controls (data not shown) indicating that the molecular lesion due to lpa-1 mutation did not affect the gene transcription but is likely to be caused by a sequence mutation in ZmMRP4 coding sequence. The homozygous lpa-1/lpa-1 were not able to germinate probably because the mutation causes approximately a 10-fold increase in the amount of free phosphate and a reduction of about 88% of PA while we did not observe significant alteration in total P amount (Figure 1). These data are in agreement with previous studies indicating a limit for PA reduction compatible with maize seed viability of about 55–65%, in fact the viable lpa-1 mutant has a reduction of PA of about 65% (Raboy et al. 2001), whereas all the not viable mutants exhibiting a higher reduction in PA such as lpa-241 (Pilu et al. 2003) and lpa-1/mm (Shi et al. 2007) have a PA reduction of about 90%. The same behavior was observed in studies conducted on rice lpa2 mutant, homologous orthologous of the maize lpa1 mutant (Xu et al. 2009), whereas similar (from a biochemical point of view) barley lpa mutations showing more than 90% of reduction in seed phytate were viable (Larson et al. 1998; Rasmussen and Hatzack 1998; Bregitzer and Raboy 2006). However, to our knowledge, it has not yet been demonstrated if these barley mutants are caused by lesions in MRP transporter, in fact none of them has been characterized from a molecular point of view. Taken together, these data emphasize the important role of MRP transporter encoded by lpa1 gene, a null allele of ZmMRP4 should be lethal or showing strong negative pleiotropic effects as homozygous and a copy, also intermediate or hypomorphic, is fundamental for seed viability. The lethality of lpa-1 as homozygous can be overcome by embryo rescue and in vitro cultivation, in this way we obtained mature plants able to be selfed and produce homozygous HIP ears (Figure 2A,B). The mutants obtained exhibited pleiotropic effects related to the mutation such as slow growth rate and alteration in the leaves; in addition, they were not able to produce a functional primary root. Experiments conducted on MS medium with added plant growth regulators (IAA and GA) did not show any significant result (data not shown). We can conjecture that drought stress (one of the major negative pleiotropic effect affecting the lpa mutants) affecting the lpa-1 mutant in the field (data not shown) could be caused by an alteration of the mature root system, and we are testing this hypothesis.

The histological analysis confirmed the defect in the primary root and showed that homozygous lpa-1 embryos were smaller than wild-type controls (Figure 2D,E). We noticed alterations in the root primary meristem of the

**Discourse**

PA in grains is not only the major storage form of P but it is considered an antinutritional factor for human and other monogastric animals and it is also involved in environmental problems of pollution. For these reasons, the lpa character and the study of the PA biological pathway represent an objective for crop genetic improvement programmes (Raboy 2009).

So far in maize, 3 lpa loci have been identified: lpa1 (Raboy et al. 2000; Pilu et al. 2003), lpa2 (Raboy et al. 2000), and lpa3 (Shi et al. 2005). A common character of these mutations is the increase of the free P inorganic fraction in the seed, co-occurring with a proportional decrease of PA and without changing the total P amount (Raboy et al. 2000; Raboy 2009). For this reason, we based the detection of lpa mutant and the subsequent genetic studies on the HIP phenotype identification.

In this work, we present data regarding an lpa mutant obtained by chemical mutagenesis, we isolated a recessive lpa maize mutant, named provisionally lpa*-7, exhibiting a composition in P fraction in the kernel (Figure 1) compatible with a mutation of lpa1 class. Data collected from molecular (Supplementary Figure 2) and genetic (Supplementary Figure 3) analysis indicated the inheritance of lpa*-7 as a recessive monogenic mutation, thus we renamed the new lpa allele as lpa-1. Because it is known that maize lpa alleles can be affected by epigenetic events at
mutant embryo: the ground meristem and the procambium (between the root apical meristem and the shoot primordia) exhibited less organized structures (Figure 2F,G). This could be the main cause of lack of germination in lpa1-7 seeds even if the strong reduction in the size of the embryo could represent a general metabolic suffering caused by perturbation of inositol-derived compounds that are involved in a huge number of fundamental plant processes such as auxin transport, signal transduction, and membrane composition (reviewed by Stevenson et al. 2000). Furthermore, PA has a strong antioxidant activity tackling the formation of reactive oxygen species avoiding ageing-related damage to seed embryos (Graf and Eaton 1990; Doria et al. 2009).

Hence, the lpa1-7 mutation produced a hypofunctional MRP transporter, and although this lesion is too severe to guarantee survival in the early stage of seedling development, in vitro cultivation enabled us to overcome this phase. The recovery can also support the hypothesis of a partial redundancy of the activity of the ZmMRP4 gene due to the presence of at least one homologous paralogous gene (Cerino Badone et al. 2010): ZmMRP4 could be the major InsP transporter in the kernel, whereas in the adult plant, this role may be partially carried on by other transporters.

This last conjecture is supported by the work of Panzeri et al. (2011) in common bean, where a mutation in PmMrp1 MRP transporter, homologous to ZmMRP4, is able to confer the lpa phenotype to the seeds without any negative pleiotropic effects on the plant. The authors outlined a plant localized functional complementation from another paralogous MRP transporter, PmMrp2, capable of limiting the effects of the mutation only to within seeds. Thus, alteration in germination rate and emergence are widespread pleiotropic effects of strong lpa mutations in several crops (Raboy et al. 2000; Pilu et al. 2005; Raboy 2009; Maupin et al. 2011) except for the leguminous common bean (Panzeri et al. 2011).

Another effect referable to pleiotropic effects in mature plants of lpa1-7 homozygous is the pale-green stripes aspect of the leaves mimicking the green stripes 2 (gs2) mutation (Neuffer et al. 1997b) (Figure 2H,I) and a decrease in trichomes length coming with an increased trichome density on leaf margins (Figure 3A,B). Trichomes or hairs are present on the leaves of almost all plants acting as physical and chemical defenses against insect attack, they are also involved in several other leaf characteristics such as hydro repellency, reflective properties, and reduction of water loss due to transpiration (Moose et al. 2004). The maize leaves produce 3 distinct types of trichomes, the bigger are the macrohairs that are considered as a marker for adult leaf identity and are regulated from macrohairstless1 (mhl1) gene (Moose et al. 2004): we can conjecture that in some way the perturbation of PA synthesis could modify the expression of this gene or other genes involved in the development of the trichomes.

In conclusion, in this work, we reported the isolation and characterization, under several aspects, of lpa1-7, a maize low phytic 1 nonepigenetic allele showing a monogenic recessive inheritance; furthermore, this mutation is lethal in field conditions in the homozygous state. We demonstrated the possibility to overcome its low germination in order to obtain plants from homozygous seed by embryo rescue. This could be a useful tool for future investigations regarding the PA pathway in lpa mutants and the MRP transporter involved. Additional work will be necessary for a better characterization of the negative pleiotropic effects associated with the lpa1-7 rescued plants.

 Supplementary Material
Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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