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Study of *low phytic acid 1* locus in maize

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GENERAL INTRODUCTION AND THESIS SUMMARY

About phosphorus

Food production, coming from modern intensive farming systems, is dependent on constant supply of inputs, such as nitrogen, phosphorus and potassium. The phosphorus used in agricultural processes is mostly obtained from rock phosphate, a non-renewable resource that has no substitutes (Cordell *et al.* 2009, Elser and Bennett 2011).

The price of phosphate rock rose up in the last years but the demand continues to increase; the easy mineable deposit areas are limited and geographically concentrated in a small number of countries (China, Morocco and USA), it was recognized a reduction in quality of reserves, coming with an enhancement of the cost of extraction, processing and shipping (Cordell *et al.* 2009). The rock phosphate is a finite resource and some authors outlined its depletion in the next century (Cordell *et al.* 2009; Smil 2000).

Per capita use of phosphate fertilizer is enhanced by changes in human diets; characterized by an increase in animal products that requires more feed crops cultivation (Figure 1). Another boost in phosphate fertilizer P consumption could be inducted by the introduction of bio-energy crops, if these crops will be cultivated on lacking in nutrient, marginal land (Schröder *et al.* 2011).

Regarding phosphorus, the food production and consumption chain are not efficient, only one-fifth of the used P reaches the final consumers (Cordell *et al.* 2009). It has been estimated that nearly 50% of elemental P used yearly in global crop production activities is accumulated in the seeds in a storage form as phytic acid (PA) (Figure 1) (Lott *et al.* 2000).

Seed are an important component of food and feed, but the capability of animals, non-ruminants in particular, to use the phosphorus from PA is limited (Figure 1) (Raboy 2009).

Livestock farmers in industrialized countries use for animal nutrition naturally high P concentrations feed or even add P salts to feed such as dicalcium phosphate; 5% of globally P demand is for feed additives (Schröder *et al.* 2011).
Taken together, these factors and future perspectives encourage a way toward the phosphorus recovering and reduction in demand and losses for crops and livestock, contributing in a more sustainable agriculture (Cordell *et al.* 2009).

About phytic acid

Phytic acid (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 phytic acid (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 (Figure 1) (Labouré *et al.* 1993).
PA forming mixed salts with mineral cations is mainly 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 reduction in their nutritional value. Furthermore the undigested phosphorus contained in excreted phytin can contribute to water pollution and algal proliferation (eutrophication) (Figure 1) (Raboy 2009).
There are several effective approaches to tackle the problems casused by PA presence in food and feed.
Phytase industrially produced can be added and the enzyme activity release inorganic phosphate for animals use, by this way the P is supplied by the seed component and the P excreted is reduced. From engineered crops it is possible to obtain seeds characterized by high levels of phytase enzyme content or low PA

amount. It is also possible to take advantage of *low phytic acid* mutations isolated in several crops (Raboy 2009).

About low phytic acid crops

The negative effects of PA have led to breeding programmes which have the aim of reducing its 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 on monogastric animals using several *lpa* crops, mutant *lpa* grain supply more available P than wild type. For this reason it is not required P integration or phytase addition; furthermore the presence of P in wastes is reduced (Mendoza *et al.* 1998; Hambidge *et al.* 2004, 2005; Raboy 2009).

The *lpa* mutations can be classified into three 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 synthesis) and mutations affecting the transport of phytic acid to the vacuole (Raboy 2009; Panzeri *et al.* 2011).

In several crops *low phytic acid* 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 (Wilcox *et al.* 2000; Hitz *et al.* 2002, Yuan *et al.* 2007), in wheat by chemical mutagenesis (Guttieri *et al.* 2004), in common bean by chemical mutagenesis (Campion *et al.* 2009), in rice by physical and chemical mutagenesis (Larson *et al.* 2000; Liu *et al.* 2007), in pea by chemical mutagenesis (Warkentin *et al.* 2012).

In maize three *low phytic acid* mutants have been isolated: *lpa1* (Raboy *et al.* 2000; Pilu *et al.* 2003a) and *lpa2* (Raboy *et al.* 2000) by chemical mutagenesis, *lpa3* by transposon tagging (Shi *et al.* 2005).

Compared to 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.* 2003a). The *Lpa1* gene encodes for *ZmMRP4* (accession number EF586878) a multidrug-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 phytic acid accumulation and viability of seeds. *low phytic acid* 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 (Meis *et al.* 2003; Pilu *et al.* 2005; Bregitzer and Raboy 2006; Guttieri *et al.* 2006; Doria *et al.* 2009; 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 signalling and developmental pathways, like DNA repair, RNA editing, chromatin remodeling and control of gene expression (Raboy 2009). Furthermore phytic acid exhibits, by its ability to chelate iron, a potent antioxidant activity, avoiding the formation of reactive oxygen species (Graf *et al.* 1984, 1987, 1990).

Summary of the thesis work

With the aim to isolate new maize *low phytic acid* mutants mutagenesis treatment were performed with EMS (ethyl-methanesulfonate) (Neuffer 1994). Since wild type mature maize seeds contain high amount of phytic phosphate and low free phosphate content, we screened the mutagenized population looking for seeds containing high levels of free phosphate (HIP phenotype), a typical feature of *lpa*.

300 M_2 families obtained by EMS seed-treating and 600 M_2 families obtained by EMS treating-pollen were examined. The screening was carried out on extracts from milled seeds by titration of free phosphate using the molybdate staining.

In previous studies a single recessive *lpa* mutation (originally named *lpa241* and obtained by EMS pollen-treatment mutagenesis) was isolated and described, it was allelic to the *lpa1-1* mutant, and was consequently renamed *lpa1-241* (Pilu *et al.* 2003a, 2005).

A first evidence of non-Mendelian inheritance of *lpa1* trait came from the appearance of unexpected free phosphate phenotypes in *Lpa1/lpa1-241*. When heterozygous families were selfed, we observed an overall increase of the mutant phenotype ratio due to the appearance of weak and intermediate phenotype, not consistent with a monogenic recessive mutation. This phenomenon can be explained with a partial *Lpa1* allele silencing caused by trans interaction with the paramutagenic *lpa1-241* allele.

We performed genetic and molecular analyses of the *lpa1-241* mutation that indicate an epigenetic origin of this trait, that is, a paramutagenic interaction that results in meiotically heritable changes in *ZmMRP4* gene expression, causing a strong pleiotropic effect on the whole plant. The use of a 5-Azacytidine (a demethylating agent) treatment provided data suggesting an association between gene methylation and the *lpa1-241* phenotype. To our knowledge, this is the first report of a paramutagenic activity not involving flavonoid biosynthesis in maize, but regarding a key enzyme of an important metabolic pathway in plants.

We isolate a new maize *low phytic acid 1* mutant allele obtained by chemical EMS seed mutagenesis. We performed the allelism test with two other *lpa1* mutants: *lpa1-1* and *lpa1-241*, our mutant failed to complement these mutants. This mutant, named *lpa1-7*, 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. We conducted two experiments to ascertain the nature of *lpa1-7* mutation (gene silencing vs. sequence mutation), we analyzed the *ZmMRP4* gene expression and we performed a 5-Azacytidine (a demethylating agent) treatment of the seeds. The gene expression analysis of *ZmMRP4* did not reveal significant variations between the mutant and the wild type and the 5-Azacytidine treatment did not show differences compared to untreated controls indicating that the molecular lesion due to *lpa1-7* mutation did not affect the gene transcription but is likely to be caused by a sequence mutation in *ZmMRP4*. We also performed physiological analysis, histological observations and considerations regarding the effects of the *lpa1* mutations on the plant.

Pigmented maize contains anthocyanins and phenolic compounds which are phytochemicals synthesized in the plant by secondary metabolism; although these compounds are considered as non-nutritive, in these years the interest in antioxidant and bioactive properties has increased due to their health benefits (Stintzing and Carle 2004, Espìn *et al.* 2007, Toufektsian *et al.* 2008). Anthocyanins are water soluble secondary metabolites belonging to the class of flavonoids and they play important roles in several aspect of plant biology. The anthocyanins are present in the vacuole in a glycosilated form and their colour is influenced in part by the pH of this compart. (de Vlaming *et al.* 1983) In maize they are synthesized by a complex pathway made up of more than 20 genes, and regulated by two classes of transcription factors: *r1/b1* bHLH genes and *c1/pl1/p1* MYB gene families (Chandler *et al.* 1989; Dooner *et al.* 1991; Pilu *et al.* 2003b).

Our aim is the constitution of maize inbred lines carrying low phytic acid mutations together with regulatory genes pushing the anthocyanin accumulation in the kernels and seedlings. In this way they can compensate the leak in antioxidant activity due by the reduction in PA (an antioxidant compound) induced by the *low phytic acid* mutation (Doria *et al.* 2009).

Plants heterozygous for *lpa1-241* and homozygous *lpa1-1*, in the same background (B73 line), were used as donors in crosses with the plants (W22 line) carrying *R-sc* allele of *R1* (*colored1*) gene (Kermicle 1984) and *Sn:bol3* gene (Pilu *et al.* 2003b) .

We found that the *lpa1-241* line is able to alter the accumulation of anthocyanins in kernel tissues. The anthocyanins, are present in the vacuole where their colour is dependent on the pH. In maize the anthocyanins are cytoplasmically synthesized molecules probably transported in the vacuole by *ZmMRP3* gene activity (Goodman *et al.* 2004).

We observed an interaction between the accumulation of anthocyanin pigments in the kernel and the lpa mutations. In fact the *lpa1-241* mutant accumulates a higher level of anthocyanins as compared to wild type either in the embryo (about 3.8-fold) or in the aleurone layer (about 0.3-fold) in a genotype able to accumulate anthocyanin. Furthermore, we demonstrate that these pigments are mislocalised in the cytoplasm, conferring a blue pigmentation of the scutellum, because of the neutral/basic pH of this cellular compartment. As a matter of fact, the propionate treatment, causing a specific acidification of the cytoplasm, restored the red pigmentation of the scutellum in the mutant and expression analysis showed a reduction of *ZmMRP3* anthocyanins' transporter gene expression. On the whole, these data strongly suggest a possible interaction between the *lpa* mutation and anthocyanin accumulation and compartmentalization in the kernel.

Figures

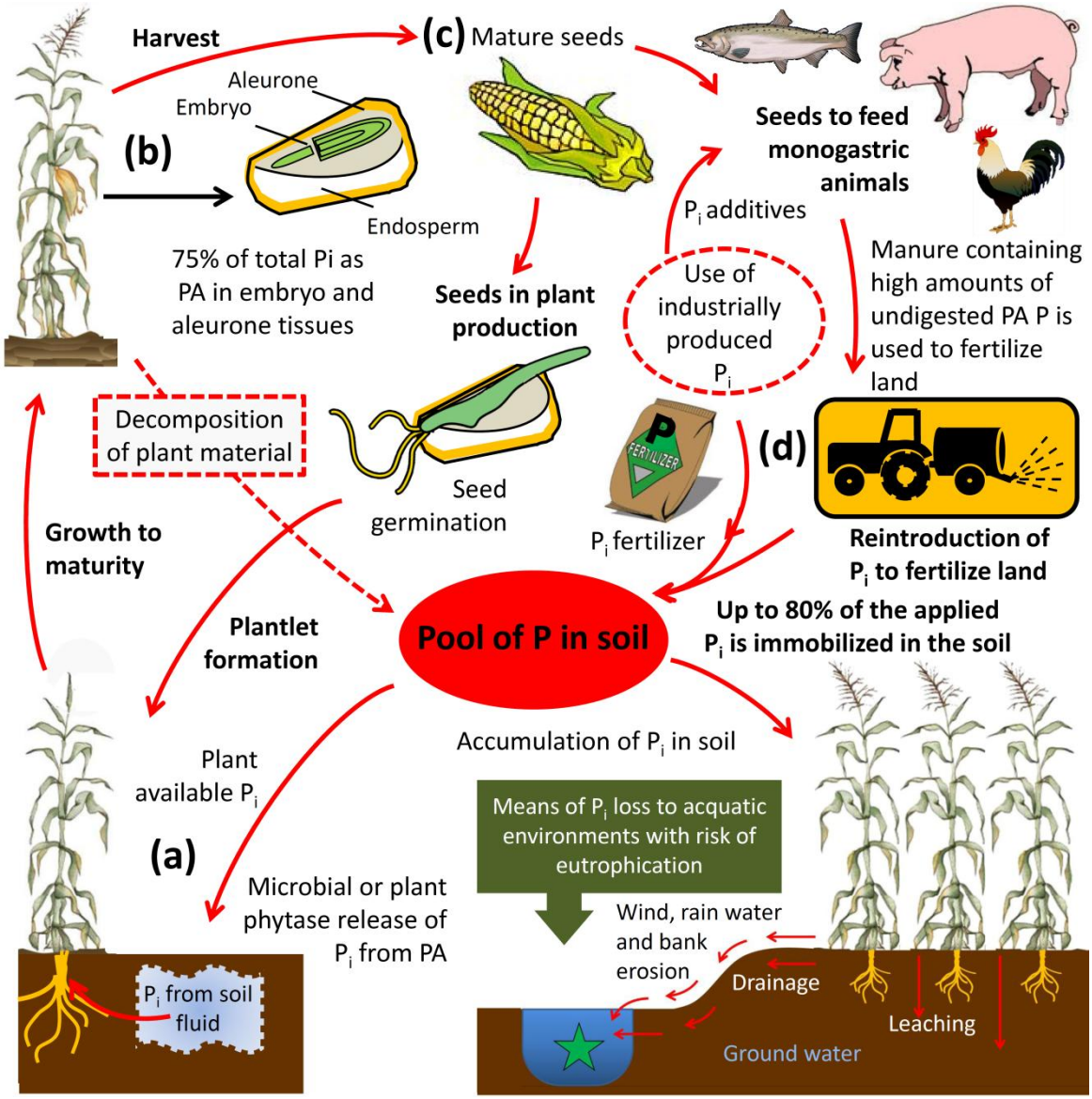

Figure 1. Phosphate and phytic acid (PA) cycles in agricultural systems.
(a) Inorganic phosphate (P_i) is absorbed by roots from the soil fluid and translocated in the plant. Only a small fraction of the P_i is available for plant requirement because the P_i is scarcely mobile and it is bond by soil particles organic and inorganic compounds.
(b) Phytic acid is the storage form of phosphate compound in seeds. Seeds can germinate or decompose like other plant material, returning P_i back to the soil after degradation of organic phosphorus compounds.
(c) Seeds can be used for plant production, as feed for livestock and food for human. The lacks in phytases activity in monogastic animals digestive apparatus causes the presence of large amounts of P in wastes as undigested phytic acid.
(d) Up to 80% of phosphorus supplied P can be rapidly fixed in forms unavailable to plants. For this reason in order to ensure crop productivity, P_i is often applied in excess. The application of P (coming from fertilizer and manure) caused accumulation in the soil. The increased phosphorus content could aggravate the P_i loss to the aquatic environment. Adapted from Brinch-Pedersen *et al.* 2002.

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A paramutation phenomenon is involved in the genetics of maize *low phytic acid1-241* (*lpa1-241*) trait

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Abstract

So far, in maize, three classes of mutants involved in phytic acid biosynthesis have been isolated: *lpa1*, *lpa2* and *lpa3*. In 2007, a gene tagging experiment performed by Shi *et al.* found that mutations in *ZmMRP4* (multidrug resistance-associated proteins 4) gene cause *lpa1* phenotype.

In previous studies, we isolated and described a single recessive *lpa* mutation (originally named *lpa241*) which was allelic to the *lpa1-1* mutant, and was consequently renamed *lpa1-241*: it showed a decrease in the expression of the myo-inositol-3-phosphate synthase gene (*mips1S*).

In this work, we present genetic and molecular analyses of the *lpa1-241* mutation that indicate an epigenetic origin of this trait, i.e. a paramutagenic interaction that results in meiotically heritable changes in *ZmMRP4* gene expression, causing a strong pleiotropic effect on the whole plant. The use of a 5-Azacytidine treatment provided data suggesting an association between gene methylation and the *lpa1-241* phenotype. To our knowledge this is the first report of a paramutagenic activity not involving flavonoid biosynthesis in maize, but regarding a key enzyme of an important metabolic pathway in plants.

Key Words: maize, phytic acid, 5-Azacytidine treatment, gene silencing, paramutation.

Introduction

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phospate, or InsP6) is the major phosphorus storage form in cereal seeds as well as in plants in general (O'Dell *et al*. 1972; Raboy *et al*. 1990). The biosynthetic route begins with inositol (Ins) and can be summarized as showed in Figure 1. *Myo*-inositol, in addition to being phosphorylated to hexa*kis* phosphate (phytic acid) during seed maturation, plays a central role in several metabolic processes and in signal transduction in the plant cell (Johnson and Wang 1996; Raychaudhuri and Majumder 1996; Majumder *et al*. 1997; Raychaudhuri *et al*. 1997). Thus, the free *myo*-inositol level may influence plant growth, development and responses to variations of environmental conditions (Munnik *et al*. 1998; Stevenson *et al*. 2000).

In maize, three different recessive *low phytic acid* (*lpa*) mutants have so far been isolated (*lpa1*, *lpa2* and *lpa3*) involved in this pathway (Figure 1). They produce seeds with reduced phytic acid content and a proportionally higher level of free phosphate, while the total amount of seed P is not significantly altered (Raboy *et al*. 2000, Pilu *et al*. 2003; Shi *et al*. 2005). The High Inorganic Phosphate phenotype (HIP) associated with the *lpa1* phenotype can be quickly determined using Chen's assay (Chen *et al*. 1956), so *lpa* mutant seeds can easily be screened. Regarding *lpa1* mutants, there is evidence indicating that they map on the short arm of chromosome 1, where a *mips1s* sequence has been localized too (Raboy *et al*. 2000). Although biochemical, mapping and gene expression data suggest the *mips1s* gene as a candidate for *lpa1* mutation in maize (Raboy *et al*. 2000; Pilu *et al*. 2003; Shukla *et al*. 2004; Pilu *et al*. 2005), recent transposon mutagenesis experiments found that a novel gene designated *ZmMRP4* (accession number EF586878), coding a multidrug-associated-protein (MRP) mapping near the *mips1s* sequence, is the actual responsible for *lpa1* mutation (Shi *et al*. 2007). MRP proteins represent a subfamily of ATP-binding cassette (ABC) transmembrane transporters widespread in all eukaryotes, which in plants are involved in several functions such as xenobiotic detoxification, organic ions transport, oxidative stress tolerance (Swarbeck *et al*. 2003) and even transpiration control (Klein *et al*. 2006).

We have previously isolated a single recessive *lpa* mutation in maize, named *lpa241*, which appeared to be allelic to *lpa1-1* and was therefore renamed *lpa1-241*. As independently found for both *lpa1-1* and *lpa1-241* alleles, *mips1S* gene expression was reduced in developing seeds (Pilu *et al*. 2003; Shukla *et al*. 2004), but in both alleles no molecular lesions in the gene coding region were found (Shukla *et al*. 2004; Pilu *et al*. 2005).

Moreover, *lpa1-241* plants exhibit a variety of morphological and physiological alterations related to the *lpa* mutation and whose extent appears linked to the phenotype expression. In particular, it has been shown that negative pleiotropic effects lead to lethality in those individuals showing less than 20% of the wild type phytate amount (Pilu *et al*. 2005). Genetic data concerning the heredity of the *lpa1-241* trait suggest that an epigenetic phenomenon called paramutation might be involved in this trait (Pilu *et al*. 2005).

Epigenetic regulation is associated with mitotically and/or meiotically heritable changes in gene expression occurring without changes in DNA sequence. These phenomena are common in eukaryotes and control a number of processes, such as, for example, development, imprinting, transposons and viral sequences silencing, as well as transgene silencing (Martienssen 1996; Wolffe and Matzke 1999).

Paramutation is a particular epigenetic phenomenon in which an allele (named paramutagenic) is capable of heritably silencing another allele (paramutable) in trans. Alleles not participating in paramutation are called neutral. So far, paramutation in maize has been studied at four loci: *r1*, *b1, p1* and *pl1,* all involved in the regulation of anthocyanin and flavonoid biosynthesis (reviewed in Chandler *et al*. 2000). For these genes, reduced pigmentation linked to the paramutated allele correlates with reduced mRNA level and in the case of *b1* locus is associated with RNAi phenomena (Das and Messing 1994; Lund *et al*. 1995; Chandler *et al*. 2000; Sidorenko and Peterson 2001; Della Vedova and Cone 2004; Chandler and Stam 2004; Stam and Scheid 2005; Alleman *et al*. 2006; Chandler 2007).

In 1956 Alexander Brink first described paramutation in maize occurring at *colored1* (*r1*), a complex locus encoding *myc*-homologous transcription factors that regulate genes involved in the anthocyanin biosynthetic pathway. *R-r:standard (R-r:std)* is a paramutable allele which produces dark pigmentation of the aleurone. When crossed with paramutagenic allele *R-stippled* (*R-st*), in the following generation *R-r:std* shows a variably reduced pigmentation (Brink, 1956). The silenced allele (designated *R-r'*) is heritable and, like *R-st,* is capable of weak paramutagenic activity for some generations (Brown and Brink, 1960). However *R-r'* reverts to normal expression (*R-r*) over subsequent generations if is no more exposed to paramutagenic allele *R-st*. In general paramutable alleles of *b1* and *pl1* loci are unstable, spontaneously changing to the silenced state with high frequency while *p1* and *r1* epigenetic states are very stable (Chandler *et al*. 2000; Sidorenko and Peterson 2001).

Although molecular mechanisms underlying these paramutation phenomena are not fully explained, in some cases specific DNA sequences such as repeated sequences and/or gene structures are known to correlate with the paramutagenic behaviour of alleles**.** Paramutation, as well as other gene silencing phenomena, has been found to correlate with changes in DNA methylation and in some cases in chromatin structure**.**

In this work, we present genetic and molecular analysis indicating that an epigenetic event like paramutation may occur at the *lpa1* locus: in fact *lpa1-241* allele exhibit paramutagenic activity vs the paramutable B73 *Lpa1* allele. To our knowledge this is the first report of a gene-silencing phenomenon causing lethality in maize.

Materials and Methods

Nomenclature

In classical paramutation, silenced (paramutated) alleles are designated with an apostrophe (e.g. *Pl'*, *B'*, etc.). In this paper we use apostrophes to distinguish between normally expressing alleles and partially silenced *Lpa1* alleles. Thus, *Lpa1*

alleles have an apostrophe when exposed in trans to *lpa1-241* allele for one generation. After two generations of exposure to paramutagenic *lpa1-241,* the allele has two apostrophes (*Lpa1''*) and so on. When allele genotype can be determined using a *ZmMRP4* allele specific PCR based molecular marker, a suitable subscript indicating the inbred line is added: alleles from ACR inbred plants are named $Lpa1_{ACR}$, while alleles from B73 are $Lpa1_{B73}$.

Genetic stocks

The *lpa1-241* mutant was originally isolated from the M_2 progeny of chemically (ethyl methane sulphonate, EMS) mutagenized populations (Pilu *et al*. 2003). Plants heterozygous for *lpa1-241* in the ACR inbred line were used as donors in crosses with the B73 inbred line for five generations and selfed. BC_5F_2 seeds from both pedigrees were used for quantitative analysis of free phosphate, 5-Azacytidine treatment, *mips1S* gene expression, *ZmMRP4* gene expression and methylation analysis. Each selfed generation was also crossed with the homozygous *lpa1-1* mutant in B73 background, and seeds were tested for free phosphate content and phenotype segregation; *mop1-1* stock (*B' mop1-1/mop1-1*) was provided by the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu/). The *lpa1-1* mutant stock was kindly provided by Dr. Victor Raboy, USDA ARS, Aberdeen, ID, USA.

Quantitative free phosphate assay (detection of HIP phenotype)

Individual or pooled seeds were ground in a mortar with a steel pestle and 100 mg of the resulting flour was extracted with 1 ml 0.4 M HCl for one hour at room temperature. Samples were mixed briefly and 100 µl were removed and supplemented with 900 µl 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 (Chen *et al*. 1956). In these conditions, if phosphate is present, a blue coloured phosphomolybdate complex forms in 1-2 hours. After 1 hour at room temperature, the free phosphate content was quantified via use of a spectrophotometer (λ = 650 nm) or evaluated by visual inspection. A KH_2PO_4 solution was used as phosphate

standard. To test the seeds for HIP phenotype without interfering with their capacity of growth, a small amount of scutellum flour was carefully obtained from a single incision by a hand drill. The flour was extracted in microtiter with 200 µl 0.4 M HCl for one hour at room temperature and then 800 µl of Chen's reagent was added. After one hour a Strong HIP phenotype could be detected by visual inspection.

lpa1 allele molecular genotyping

Allele genotyping was performed by PCR amplification of a *ZmMRP4* sequenced gene portion. Allele-specific primers were designed on a two nucleotides insertion polymorphism found in the ACR *ZmMRP4* 10th intron. ACR specific primer was ZmMRP4+6092Ra (5'- AATCAAGACGATGAGAAAAGTTAT-3'), while B73 specific primer was ZmMRP4+6092Rb (5'-AATCAAGACGATGAGAAAAGTTC-3'). ACR allele specific amplifications were performed in a reaction mix containing an aliquot of genomic DNA, 1X Green Go Taq buffer, 2.5 µM $MgCl_2$, 0.2 µM each of dATP, dCTP, dGTP and dTTP, 0.3 µM of reverse ZmMRP4+6092Ra ACR specific primer, 0.3 µM of forward ZmMRP4+5590F primer (5'-TGGGAATGTGGTTTCTTAATGC -3') and 0.025 unit of Go Taq Flexy DNA polymerase (Promega, Madison, WI, USA), in a final volume of 25 µl. 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 63° for 1 min, extension at 72° for 1.5 min. Extension at 72° for 5 min was performed to complete the reaction. The ACR allele specific amplification product is 498 bp long. B73 allele specific amplifications were performed in a reaction mix identical to that of ACR allele specific amplifications, except that 0.3 µM of ZmMRP4+6092Rb B73 specific primers was used instead of ZmMRP4+6092Ra. The reaction mix underwent an initial denaturation step at 94°C for 2.5 min, 36 cycles of denaturation at 94 °C for 45 s, annealing at 65° for 1 min, extension at 72° for 1.5 min. Extension at 72° for 5 min was performed to complete the reaction. The B73 specific amplificate is 503 bp long. Amplification products were visualized on 1.5% (w/v) agarose gels with ethidium bromide staining.

5-Aza-2′-Deoxycytidine (Azacytidine) treatment

Mature dry seeds were sterilized with 5% sodium hypochlorite for 15 min and then incubated with 20 ml of 30 μM 5-aza-2′-deoxycytidine (Sigma, product No. A3656, St. Louis, Mo, USA) solution in rotating flasks at 28° C for 15 hours (in order to obtain mature plants), or at 30° C for 18 hours (for embryo rescue and seedlings analysis). Control seeds were incubated with 20 ml deionised water under the same conditions.

Embryo rescue

5-Azacytidine treated embryos were removed aseptically and transferred to Murashige and Skoog salt mixture (pH 5.6) (Sigma, product no. M5519) containing 2% sucrose, solidified with 0.8% agar (Plant agar, Duchefa, Haarlem, The Netherlands). Cultures were incubated in a growth chamber at 25°C with a 18/6 light/dark photoperiod. Seedling elongation was measured after 6 to 14 days and shoot tissue was sampled and stored at –80° for subsequent DNA and RNA extraction. The light source consisted of four cool white (F36T12/CW/HO) fluorescent lamps from GTE SYLVANIA (Lighting Products Group, Danvers, MA). This experiment was conducted on a total of 20 treated *lpa1-241*/*lpa1-241*, 23 untreated *lpa1-241*/*lpa1-241*, 33 treated wild type, 38 untreated wild type, 26 treated *lpa1-1/lpa1-1* and 26 untreated *lpa1-1/lpa1-1* individuals.

RT- PCR expression analysis

Total RNA was extracted from frozen shoots of 6-7 days old wild type, or 14-15 days old selected Strong HIP phenotype seeds using the method described by van Tunen *et al*. (1988). Reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect *mips1S* and *ZmMRP4* gene transcripts. First strand cDNA was synthesized with an oligo (dT) primer from total RNA extracted from shoots. All RNA samples were treated with DNase (1 unit/μg) before cDNA synthesis. First-strand cDNA was used as the template for PCR amplification. Amplification reactions containing an aliquot of cDNA synthesized from 5 μg of total RNA, 1X

Green Go Taq buffer, 2.5 µM $MgCl_2$, 0.2 µM each of dATP, dCTP, dGTP and dTTP, 0.3 µM of each primer and 0.025 unit of Go Taq Flexy DNA polymerase (Promega) were performed in a final volume of 25 µl. The reaction mix underwent 34 cycles of denaturation at 94 °C for 45 s, annealing at 62° for 1 min, extension at 72° for 1.5 min. Extension at 72° for 5 min was performed to complete the reaction. A set of primers specific for the *orange pericarp 1* (*orp1*) gene, which encodes the *β*-subunit of tryptophan synthase (Wright *et al*. 1992), was used to standardize the concentration of the different samples. *orp1* specific sequences were amplified using the following primers: The upstream primer, 5'-AAGGACGTGCACACCGC-3', and downstream primer, 5'-CAGATACAGAACAACAACTC-3'. The length of the amplified product was 207 bp. Several cycles of successive cDNA dilutions and *orp1* amplification were done in order to obtain similar amplification signals in the different samples. For mRNA detection of the *mips1S* gene under analysis, the following specific primer sets were used: Zm1302 (upstream primer 5'- GCTCTTGGCTGAGCTCAGCA -3') and Zm1580 (downstream primer 5'-GTTCCCTTCCAGCAGCTAAC-3'). The amplified product was 279 bp. *ZmMRP4* mRNA detection was conducted with specific primers designed on *ZmMRP4* genomic sequence (Shi *et al*. 2007): ZmMRP4+5135F (upstream primer 5'- tcatggtgtaagttgtatgtttc -3') and ZmMRP4+6206R (downstream primer 5'- cctctctatatacagctcgac -3'). A 677 bp amplificate is obtained after 33 cycles of denaturation at 94 °C for 45 s, annealing at 60° for 1 min, extension at 72° for 1.5 min. Final extension at 72° for 5 min was performed. Each expression analysis was conducted on RNA extracted from five individuals, in three replicates at least.

PCR products were loaded on 2% (w/v) agarose gels and visualized by ethidium bromide staining under UV light.

Results

Inheritance of *lpa1-241* trait: lpa1-241 allele may cause partial *Lpa1* allele silencing.

In order to quickly follow the *lpa1* trait segregation, we scored for the seed free inorganic phosphate content using Chen's assay performed in microtitre plates (Chen *et al*. 1956; Raboy *et al*. 2000; Pilu *et al*. 2003). We defined four phenotypic classes corresponding to the level of seed free inorganic phosphate expressed as mg of atomic P per g of flour. These classes, wild type (0–0.3), weak (0.3–0.5), intermediate (0.5–1.4), strong (>1.4), are easily scored by visual inspection if the assays are performed in microtiter plates (Figure 2). Furthermore we used the *ZmMRP4* gene sequence data to produce an allele-specific PCR-based molecular marker to discriminate the *lpa1-241* ACR allele from *Lpa1* B73 allele and follow the alleles segregations showed in Figure 3.

As shown in Figure 3 where we report a schematic pedigree of the *lpa1-241* trait, the original *lpa1-241* mutation event occurred in an ACR inbred line (Figure 3, arrow 1). The mutant phenotype was observed in F_2, where the strong HIP class segregated 1:3 as expected for a recessive mutation (Figure 3, cross 2). In this cross, heterozygotes showed a weak phenotype although, as shown in our previous work, crosses to wild type ACR plants resulted in 100% wild type progeny (Figure 3, cross 3). Thus, we originally speculated that the mutation was not completely recessive (Pilu *et al*. 2003) and later on conjectured (Pilu *et al.* in 2005) there might be some kind of allelic interaction such as paramutation causing a silencing of the wild type *Lpa1*allele. With the aim of better understanding this behaviour, we crossed heterozygous ACR *Lpa1*/*lpa1-241* families with wild type plants from the B73 inbred line and observed that only the wild type phenotype was detected in F_1 (Figure 3, cross 4). Selfing heterozygous $Lpa1_{B73}/lpa1\text{-}241_{ACR}$ plants, we obtained a segregating F_2 generation (Figure 3, cross 5) in which the phenotype classes are similar to those obtained in segregant F_2 ACR seeds.

Furthermore, in F3 seeds (Figure 3, cross 6) we observed a general decrease in the size of wild type and weak classes, associated to a general increase of intermediate

and strong classes. The segregation data regarding F_2, F_3 and F_4 families showed a consistent increase of strong/intermediate HIP phenotype which was correlated to the number of selfings (Figure 4A and Table 1). Furthermore, no progeny was obtained from *lpa1-241* $_{ACR}$ homozygotes because of the negative pleiotropic effects associated to the strong *lpa* phenotype.

This non mendelian segregation could be explained with a progressive *Lpa1* allele silencing occurring in *lpa1-241* families over subsequent generations.

To verify these data, we crossed several heterozygous plants of subsequent generation *Lpa1*$_{B73}$/*lpa1-241*$_{ACR}$, *Lpa1'*$_{B73}$/*lpa1-241*$_{ACR}$ and *Lpa1''*$_{B73}$/*lpa1-241*$_{ACR}$ to homozygous recessive stable *lpa1-1* line. In these crosses we expected a segregation ratio of 1:1 for *lpa1* phenotype as expected for a backcross of monogenic recessive mutation. However, even in this case, phenotype segregation of the progeny showed a decrease in the size of wild type and weak classes and a correlated increase of intermediate and strong classes associated to progressive exposure of the *Lpa1* allele to the paramutagenic *lpa1-241* (Table 2 and Figure 4B).

Paramutated *Lpa1* allele reverts to wild type

Segregating families carrying a partially silenced *Lpa1'* allele showed a weak phenotype (Figure 3, cross 7), but in subsequent generations of selfing, we observed a reversion to wild type in the absence of the *lpa1-241* allele.

In line with this, measuring the seed free phosphate amount in partially silenced *Lpa1'/Lpa1'* progeny (Figure 3, cross 8), we detected a progressive reversion to wild type phenotype: *Lpa1'/Lpa1'* seed phenotype from a F_2 segregant family resulted in 0.50 mg of atomic P per g of flour in average, while subsequent progeny from these homozygous *Lpa1'* plants produced seeds with lower free phosphate levels: 0.20 mg/g for F_3 and 0.14 mg/g for F_4 seeds.

The same behaviour was observed in homozygous *Lpa1''*$_{B73}$ and *Lpa1'''*$_{B73}$ progeny: three subsequent selfed generations were produced and when the average seed free phosphate content was measured in each generation, a steady decrease was observed (data not shown).

lpa1-241 exhibits silencing activity over different alleles

In order to directly detect $Lpa1_{B73}$ allele silencing, heterozygous $Lpa1_{B73}/lpa1\text{-}241$ F_1 plants were crossed with homozygous *lpa1-1*/*lpa1-1* plants in B73 background, and the seed free phosphate content of the progeny was assayed (Figure 5A). A 1:1 segregation ratio between strong and weak phenotype, indicating a partial silencing of $Lpa1_{B73}$ allele (weak phenotype), was obtained. As a control, the progeny of the cross between B73 wild type plants and B73 *lpa1-1* plants was assayed and only wild type seeds were obtained (Figure 5B).

The same experiment was conducted with plants from W64A, K6 and W22 inbred lines. A F_1 family of each genotype, $Lpa1_{W64A}/lpa1\text{-}241$, $Lpa1_{K6}/lpa1\text{-}241$, $Lpa1_{H99}/lpa1\text{-}241$, and $Lpa1_{W22}/lpa1\text{-}241$ F_1 was crossed with a B73 *lpa1-1*/*lpa1-1* family. Each cross progeny was analyzed by visual inspection and found to segregate 1:1 for strong and weak phenotype, indicating that the expression of every tested allele is reduced after one generation *in trans* with the $lpa1\text{-}241_{ACR}$ allele. As negative controls, wild type plants from each inbred line were crossed with a B73 *lpa1-1*/ *lpa1-1* family and the progeny assayed: only wild type seeds were obtained.

Lpa1 gene undergoes spontaneous silencing with high frequency

In order to estimate the frequency of spontaneous occurrence of *lpa1* mutation, B73 homozygous *lpa1-1* mutant lines were crossed with wild type *Lpa1/Lpa1* B73 plants and the progenies were screened for the strong free phosphate phenotype. We used *lpa1-1* mutation as a tester in order to detect the phenotypic expression level of possible silenced *Lpa1'* epialleles as well as to evaluate the spontaneous frequency of occurrence of *lpa1* mutation because it has a stable, strong HIP phenotype, and is viable in homozygosity (Raboy *et al*. 2000).

About 2500 F1 seeds were assayed and 13 seeds displaying strong silencing were detected, indicating novel events of *Lpa1* gene silencing. Thus the frequency of spontaneous silencing of *Lpa1* allele resulted 5.2×10^{-3}.

Lpa1 gene silencing is not affected by *mop1-1*

The wild type gene mediator of *paramutation1-1* (*Mop1-1*) is required for establishment and maintenance of paramutation at several maize loci and the *mop1-1* recessive mutation affects paramutation (Dorweiler *et al.* 2000), so we performed a test to assay the behaviour of our *lpa1* mutation. We used a genotype carrying *B'* allele so as to be able to follow in the offspring the purple plants homozygous for *mop1-1/mop-1*.

Heterozygous *Lpa1/lpa1-241* families were crossed with *mop1-1 B'* plants and the progenies were selfed. *B'/-* coloured plants present in the progeny were homozygous *mop1-1/mop1-1*. 27 coloured (*mop1-1/mop1-1 B'/-*) and 18 colourless/light (*Mop1-1/- B'/-)* plants used as control were self fertilized and their progenies assayed for HIP phenotype (Figure 6). HIP phenotype was found in both coloured and colourless plants, indicating that the *mop1-1* mutation is unable to modify the *lpa1-241* phenotype.

Seed treatment with 5-Azacytidine partially reverts HIP phenotype and reduces pleiotropic effects in *lpa1-241* mutant seedling

Two different experiments with the demethylating agent 5-Azacytidine were conducted on *lpa1-241* mutants, the first experiment concerning the study of the effect of 5-Azacytidine seeds treatment on the HIP offspring phenotype, and the second one regarding the effect of this treatment on the pleiotropic effects on the seedling caused by *lpa1-241* mutation.

A significant reduction of the phenotype strength was found in *lpa1-241/lpa1-241* individuals obtained from self-pollinated plants derived from 5-aza-2'-deoxycytidine treated seeds compared to untreated controls (Figure 7). By contrast, neither seeds produced by treated wild type B73 seeds nor homozygous *lpa1-1* seeds (data not shown) revealed significant differences in respect to untreated controls (Figure 7).

Seeds expressing the strong HIP phenotype are unable to germinate, but slow growing seedlings can be obtained if embryos are rescued *in vitro* on MS medium.

These seeds treated with 5-Azacytidine always showed a remarkable growth increase (of about 50%) compared to untreated controls of the same phenotypic class (Figure 8A). No significant difference was detected between treated and untreated *Lpa1/Lpa1* B73 (Figure 8B) or *lpa1-1* homozygote seedlings (not shown in figure).

The demethylating agent 5-Azacytidine partially restores ZmMRP4 (*Lpa1* gene) as well as *mips1s* gene expression.

After treatment of seeds with 5-Azacytidine, *mips1s* and *ZmMRP4* gene expression levels were analyzed in seedling tissues by RT-PCR.

As previously reported in our studies on *lpa1-241* mutation (Pilu *et al.* 2003), *mips1s* expression is reduced in untreated strong HIP phenotype-expressing mutants compared to the untreated wild type (Figure 8C, lane 1 vs lane 3). A detectable increase in *mips1s* expression was instead found in treated *lpa1-241/lpa1-241* strong HIP phenotype mutants compared to untreated mutants of the same phenotypic class (Figure 8C, lane 3 vs lane 4), while no detectable differences were found between treated and untreated wild type (Figure 8C, lane 1 vs lane 2). *ZmMRP4* gene expression shows a similar pattern to that of *mips1s*. Untreated mutants show a lower expression compared to untreated wild type (Figure 8C, lane 1 vs lane 3), and, again, treated mutants expression level increases after 5-Azacytidine treatment (Figure 8C, lane 3 vs lane 4).

RT-PCR analysis performed on *lpa1-1* homozygote seedlings carried out in the same experimental conditions showed no significant variation in the expression of both *mips1s* and *ZmMRP4* genes (Figure 8D).

Discussion

The *lpa1-241* recessive mutation was isolated in an EMS mutagenesis ACR inbred line showing a strong HIP phenotype in the seed. A first evidence of non-Mendelian inheritance of *lpa1* trait came from the appearance of unexpected free phosphate phenotypes in $Lpa1_{ACR}/lpa1\text{-}241_{ACR}$. When heterozygous families were selfed, we observed an overall increase of the mutant phenotype ratio due to the

appearance of weak and intermediate phenotype, not consistent with a monogenic recessive mutation (Figure 3, cross 2). This phenomenon can be explained with a partial *Lpa1* allele silencing caused by *trans* interaction with the paramutagenic *lpa1-241* allele.

To prove this hypothesis we used an allele-specific PCR-based molecular marker to discriminate the *lpa1-241* ACR allele from *Lpa1* B73 allele and follow the alleles segregations. More accurate evidences were obtained by selfing *lpa1-241*/$Lpa1_{B73}$ heterozygotes (Figure 3, crosses 5 and 6).

As observed for ACR families, in F_2, F_3, F_4 generations where *lpa1-241* allele was present, seed free phosphate levels increased, suggesting a reduction in activity of the $Lpa1_{B73}$ allele (Table 1 and Figure 4A). After some generations, selfed families reached a level of phenotype expression whose pleiotropic effects seriously impair fitness. Thus, no further progeny can be obtained from such plants. In addition, the progressive *Lpa1* silencing (*Lpa1'*, *Lpa1''*, etc.) could be also detected by crossing plants from each generation with homozygous *lpa1-1*: where a 1:1 segregation ratio is expected, we scored mutant seeds in more than 50% of the cases (Table 2 and Figure 4B).

A common aspect in gene silencing phenomena such as some cases of classical paramutation is that spontaneous silencing can occur with high frequency. We found that silenced *lpa1* alleles showing the strong HIP phenotype can spontaneously occur in B73 line with a frequency of more than 10^{-3}.

Collected genetic and phenotypic data regarding the hereditability of *lpa1-241* locus are compatible with a gene silencing phenomenon such as paramutation. So far, paramutation in maize has been studied at four loci *r1*, *b1*, *pl1* and *p1,* all involved in flavonoids and anthocyanins biosynthesis (reviewed in Chandler *et al*. 2000).

The *booster1* (*b1*) locus contains a single coding region for a transcription factor that regulates synthesis of anthocyanin pigments in many epidermal tissues. The active, paramutable *B-I* (*Booster-Intense*) allele spontaneously becomes partially silent (*B'*) with high frequency. Crossing an active *B-I* with a *B'* causes the down-regulation of *B-I*, which also acquires paramutagenic activity (Coe 1959; 1966).

In 1995, paramutation was discovered at *purple plant1* (*pl1*) locus, another anthocyanin pathway regulator. In this case, the paramutable allele *Pl-Rhoades* (*Pl-Rh*) is silenced when exposed *in trans* to its spontaneously derived, silenced paramutagenic *Pl'* allele (Hollick *et al*. 1995).

At *p1* (*pericarp color1*) locus, two spontaneous epialleles (*P-pr-1* and *P-pr-2*) were isolated. They showed moderate stability and weak paramutagenic effect on *P-rr* allele (Das and Messing 1994). In addition, a heritable, paramutagenic *P-rr'* silenced allele arises by transgene-induced silencing (Sidorenko and Peterson 2001). This locus controls the expression of phlobaphene pathway in several tissues, including pericarp and cob.

DNA methylation is known to correlate with epigenetic gene silencing and epigenetic gene silencing is known to occur by repression of transcription (TGS, Transcriptional Gene Silencing) or by affecting mRNA stability (PTGS, Post Transcriptional Gene Silencing). TGS is associated to chromatin remodelling processes such as histones modification, substitutions and DNA methylation (Grant-Downton and Dickinson, 2005), while PTGS is mediated by various classes of small RNAs (Vaucheret, 2006). Small RNA pathways also act on DNA, mediating chromatin remodelling or even sequence elimination (Vaucheret, 2006). With the aim of understanding whether DNA methylation is involved in our trait, we performed experiments with the demethylating agent 5-aza-2'-deoxycytidine. Interestingly, we found that treated seeds yielded in the next generation homozygous mutant seeds with a significant reduction, compared to control, in free phosphate (Figure 7). No significant variation was found for wild type B73 used as control or *lpa1-1/lpa1-1* seeds (data not shown). Also, selected *lpa1-241/lpa1-241* strong HIP phenotype expressing seeds treated with 5-Azacytidine showed a reduction in negative pleiotropic effects when rescued in vitro (Figure 8A), while we could not report a significant effect either on *Lpa1/Lpa1* control (Figure 8B) or on *lpa1-1* individuals (data not shown). These results strongly suggest that the *lpa1-241* allele could be silenced by means of epigenetic mechanisms involving DNA methylation. In line with this, *lpa1-1* homozygotes

were 5-Azacytidine insensitive, in accordance with the fact that a sequence mutation was found in the *ZmMRP4* gene (Shi *et al*. 2007).

As previously reported for shoot tissue (Pilu *et al*. 2003), we confirmed that *mips1S* mRNA level is reduced in *lpa1-241* mutants compared to wild type. In shoots obtained from 5-Azacytidine treated seeds we observed a detectable increase in *mips1s* mRNA level (Figure 8C). A similar pattern was found for *ZmMRP4* mRNA in the same tissues: its mRNA level seems reduced in *lpa1-241* mutant compared to wild type, while 5-Azacytidine treatment causes a slight increase (Figure 8C). These results are consistent with the hypothesis that *lpa1-241* mutant phenotype is due to epigenetic silencing of *ZmMRP4* and, either directly or indirectly, *mips1s* gene. We may hypothesize that the reduced *mips1s* mRNA level independently found in developing seeds of *lpa1-1* (Shukla *et al*. 2004) and in seeds and shoots of *lpa1-241* mutants (Pilu *et al*. 2003), could be due to a metabolic feedback caused by an excess of *myo*-inositol phosphorylated intermediates or end products, such as phytic acid itself. In fact, MRP proteins in plants are vacuolar transporters involved in detoxification of both xenobiotics or endogenous substances (Klein *et al*. 2006), thus the loss of *ZmMRP4* activity may cause a cytosolic accumulation of its substrate, which may in turn feedback inhibit *mips1s* transcription. Another possibility is that the same epigenetic silencing phenomenon acting on *ZmMRP4* gene may also silence *mips1S* gene.

Pleiotropic effects and phenotype reduction observed after 5-Azacytidine treatment can be primarily ascribed to an increase in *ZmMRP4* expression level and transport activity, which, indirectly, may promote *mips1s* transcription by removing the hypothetical metabolic negative feedback. Alternatively, demethylation might remove epigenetic silencing marks from both *ZmMRP4* and *mips1s* genomic sequences, restoring their transcription potential.

A further explanation may be that HIP phenotype and pleiotropic effects reduction in *lpa1-241* mutant after 5-Azacytidine treatment may be due to an epigenetic activation of one or multiple genes which contribute to partially overcome the *lpa1-241* mutant phenotype.

Several mutants affecting paramutation have been isolated in maize: one *mediator of paramutation1* (*mop1*) and three *required to maintain repression* (*rmr1*, *rmr2* and *rmr6*) (Dorweiler *et al*. 2000, Hollick and Chandler 2001, Hollick *et al.* 2005). *Mop1* encodes for an RNA-dependent RNA polymerase, which, together with *Rmr2* (Hollick and Chandler 2001), is required for somatic maintenance of the paramutant state of *Pl'* and *B'* but not *R'* allele. *Mop1* is also required to establish silencing at *b1* and *r1* loci (Dorweiler *et al*. 2000, Alleman *et al*. 2006), and is able to progressively reactivate a silenced *MuDR* element (Woodhouse *et al*. 2006).

Analysis of progenies obtained by crossing the *mop1-1* mutant with our lpa mutants (Figure 6) showed that *mop1-1* mutant is not involved in the maintenance of the silenced state of *lpa1-241* allele. So far, we don't know if paramutation is also affected, and we plan to do this experiment in the near future. *Mop1* is an RNA-dependent RNA polymerase possibly involved in maintaining a threshold level of some kind of silencing RNA which mediates transcriptional gene silencing (Alleman *et al*. 2006). Although 5-Azacytidine significantly reverts *lpa1-241* phenotype, *mop1* mutation cannot. This evidence indicates that the hypothetical silencing phenomenon seems somewhat similar to *r1* locus paramutation (Brink, 1956).

Further studies will be necessary to understand the mechanism of establishment and maintenance of these *lpa1* epialleles, and in particular we are planning to study chromatin structure in this region.

In conclusion, this is the first report of a paramutation phenomenon involving a fundamental metabolic pathway in maize which might be the tip of an iceberg of homology-sensing mechanisms involved in several biological phenomena, not so far fully understood, such as heterosis.

Acknowledgements

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Figures and Tables

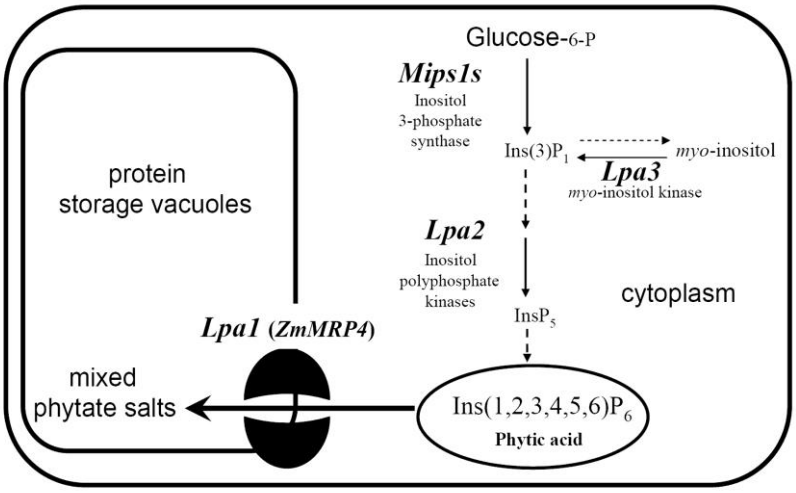

Figure 1. Schematic model of the biosynthetic pathways leading to phytic acid accumulation in protein storage vacuoles of the maize seed. Main known genes involved in Ins phosphate pathway are shown. *Mips1S* encodes for a *Myo*–Inositol(3)P_1 synthase which converts glucose-6-P to Ins(3)P_1. Ins(3)P_1 can be produced also by the action of a *myo*-inositol kinase encoded by *Lpa3* gene. *Lpa2* encodes an inositol phosphate kinase that along with other kinases leads to phytic acid, Ins(1,2,3,4,5,6)P_6, synthesis. *Lpa1* gene (*ZmMRP4*) is a transmembrane transporter hypothesised to load phytate into protein storage vacuoles.

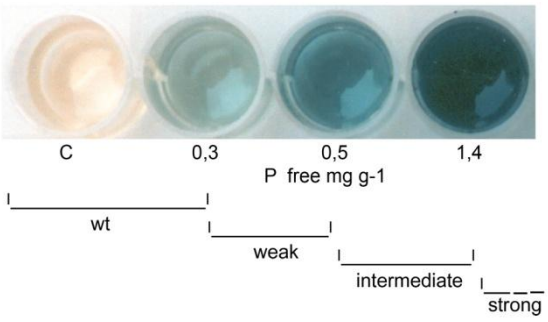

Figure 2. Assay to detect *lpa1-241* phenotypic classes. Single seeds were crushed, extracted and assayed for free P using a microtitre plate-based colorimetric assay (Chen *et al*. 1956). Classes detectable by visual inspection were: wild type (wt) 0–0.3; Weak (W) 0.3–0.5; Intermediate (I) 0.5–1.4; Strong (S) >1.4, expressed as mg of atomic P per g of flour.

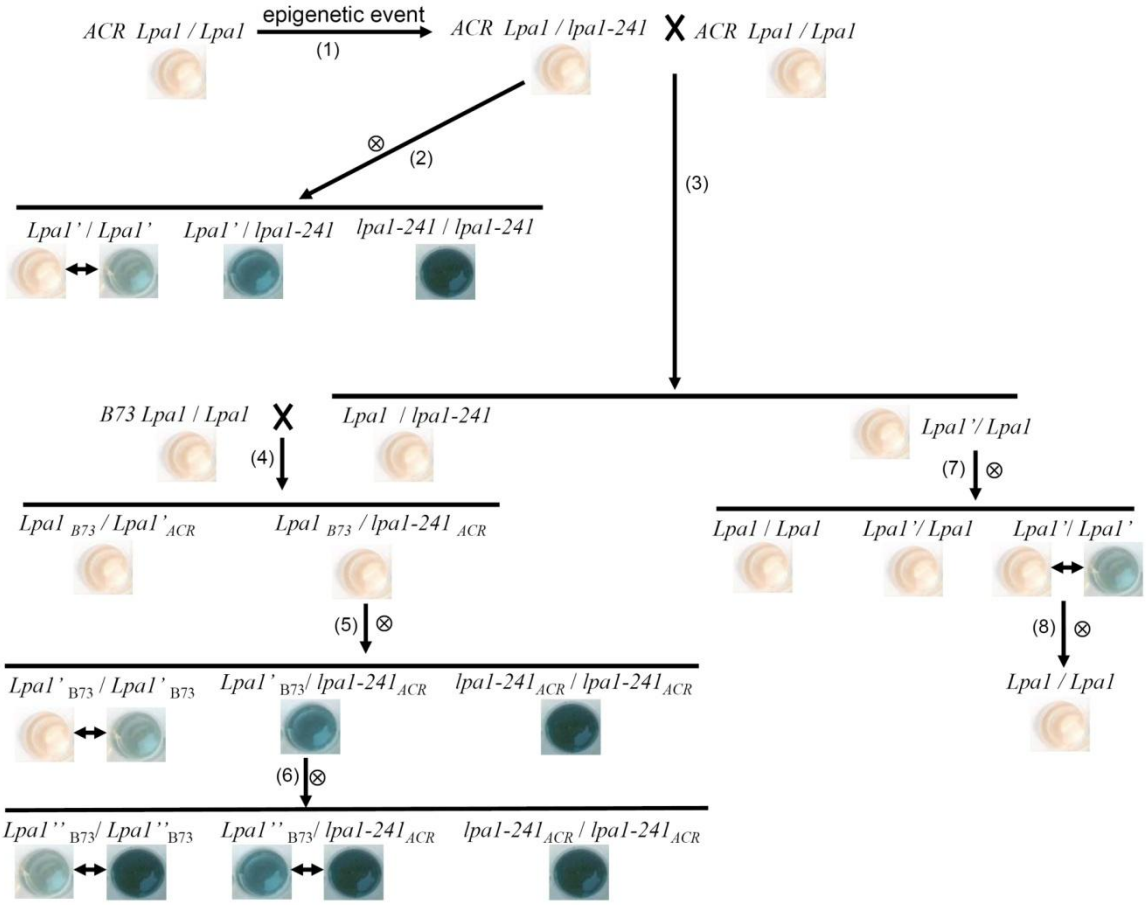

Figure 3. Diagram of *lpa1-241* mutation pedigree. A *Lpa1* wild type allele spontaneously underwent silencing in ACR inbred line (1). The event was named *lpa1-241* and its phenotype was detected in the progeny obtained by selfing *Lpa1/lpa1-241* ACR plant (2). *Lpa1/lpa1-241* ACR heterozygous was also crossed with *Lpa1/Lpa1* ACR line (3) and the heterozygous *Lpa1/lpa1-241* ACR obtained was crossed to a wild type B73 inbred line (4). Segregating phenotypes obtained in subsequent generations of selfing are shown (5, 6). Phenotypes and genotypes of the progeny of a weakly silenced allele *Lpa1'* are also shown (7, 8). A free P microtiter plate-based colorimetric assay (Chen *et al*. 1956) associated to genotype is also shown. Genotypes have been determined by specific *ZmMRP4* allele molecular marker.

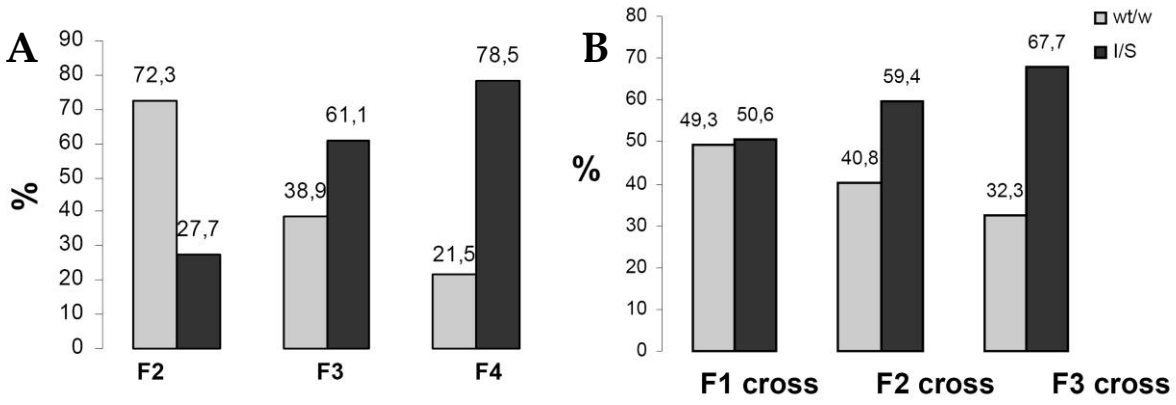

Figure 4. Changes in phenotypic class segregation ratios over generations in presence of *lpa1-241* allele. (A) Seed free phosphate phenotypic classes are presented in histogram as a percentage of occurrence in selfed heterozygous progenies, where F_2 refers to *Lpa1/lpa1-241* selfed progeny, F_3 refers to *Lpa1'/lpa1-241* selfed progeny and F_4 refers to *Lpa1''/lpa1-241* selfed progeny. (B) Histogram representation of phenotypic classes segregation ratios in the progenies of the same families crossed with a homozygous *lpa1-1* recessive mutant: F_1 cross refers to *Lpa1/lpa1-241* x *lpa1-1/lpa1-1* progeny, F_2 cross refers to *Lpa1'/lpa1-241* x *lpa1-1/lpa1-1* progeny, F_3 cross refers to *Lpa1''/lpa1-241* x *lpa1-1/lpa1-1* progeny. The sum of wild type and weak (wt/w) phenotype occurrence (gray bars) versus intermediate plus strong (I/S) phenotype occurrence (black bars) are shown. Percentage values are shown at the top of each bar.

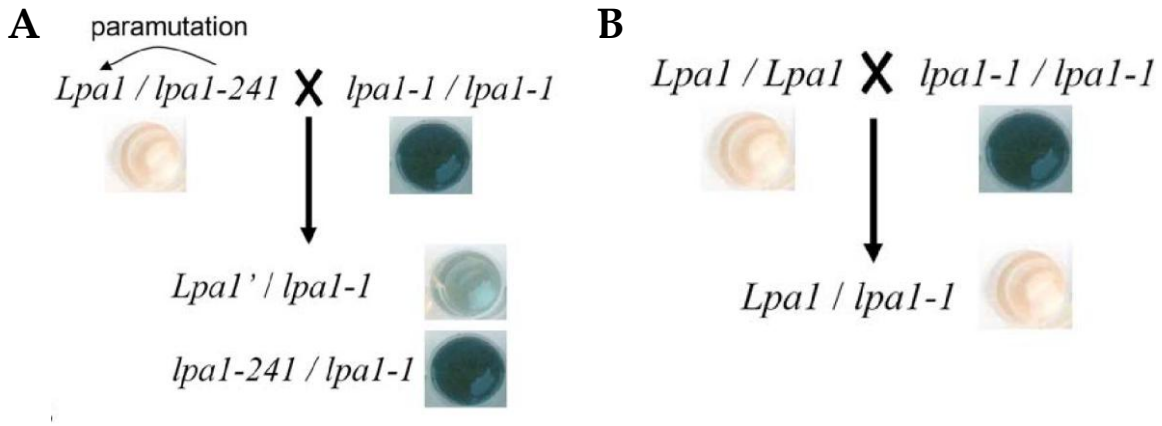

Figure 5. *Lpa1* allele silencing test after exposure to *lpa1-241* paramutagenic allele. Heterozygous *Lpa1/lpa1-241* families have been used in crosses with homozygous *lpa1-1* mutant as a test for *Lpa1* alleles activity. (A) Phenotypes obtained crossing *lpa1-1/lpa1-1* with a *Lpa1/lpa1-241* F_1 family. Curved arrow indicates the silencing effect of *lpa1-241* allele on the *Lpa1* allele causing silencing (*Lpa1'*). (B) Seed free phosphate phenotype of wild type *Lpa1/Lpa1* line crossed with *lpa1-1/lpa1-1*.

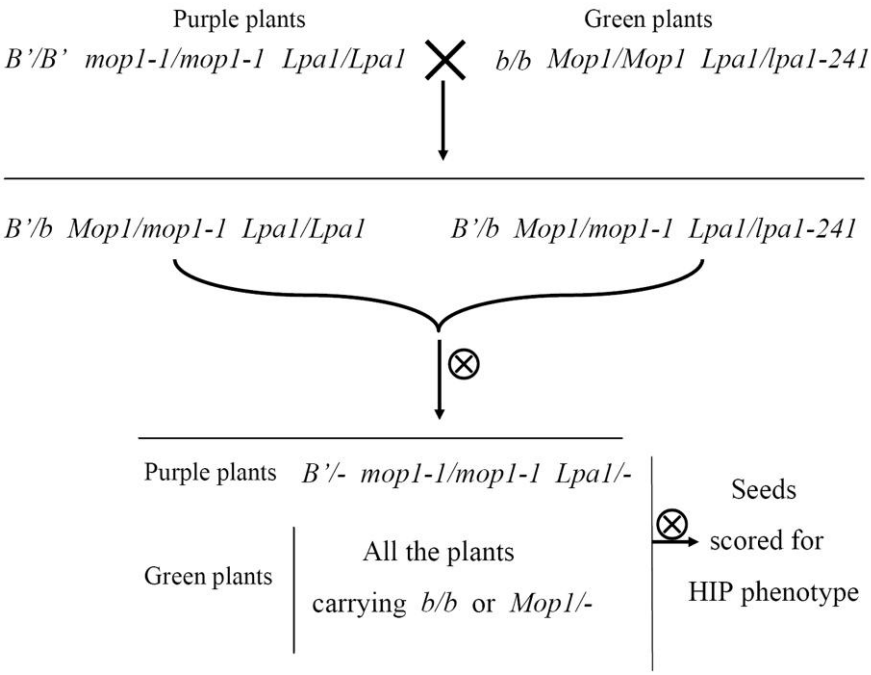

Figure 6. Diagram showing the crosses between *mop1-1* and *lpa1-241* mutants, and the genotypes tested. Homozygous *mop1-1* purple plants (*B'/B' mop1-1/mop1-1 Lpa1/Lpa1*) were crossed with heterozygous *lpa1-241* green plants (*b/b Mop1/Mop1 Lpa1/lpa1-241*), the progeny was selfed and seeds obtained were planted. Purple plants (*B'/- mop1-1/mop1-1 Lpa1/-*) were then selfed and the seeds obtained scored for inorganic phosphate content. Green plants were used as control.

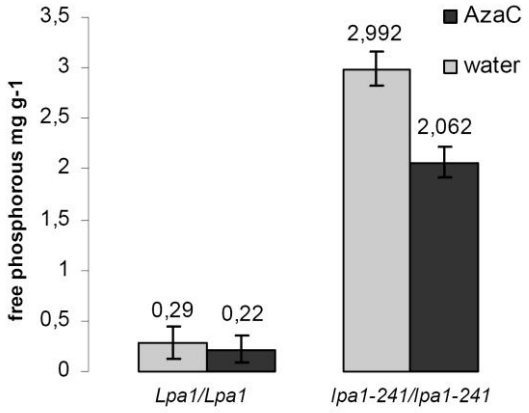

Figure 7. Seed free phosphorus content of 5-Azacytidine treated *lpa1-241* seeds progeny. Average seed free phosphorus content (mg P per g of flour) of *Lpa1/Lpa1* B73 and homozygous *lpa1-241/lpa1-241* individuals from *lpa1-241* segregating families. Black bars refer to 5-Azacytidine treated seeds; gray bars refer to untreated controls. Confidence intervals at 95% are shown.

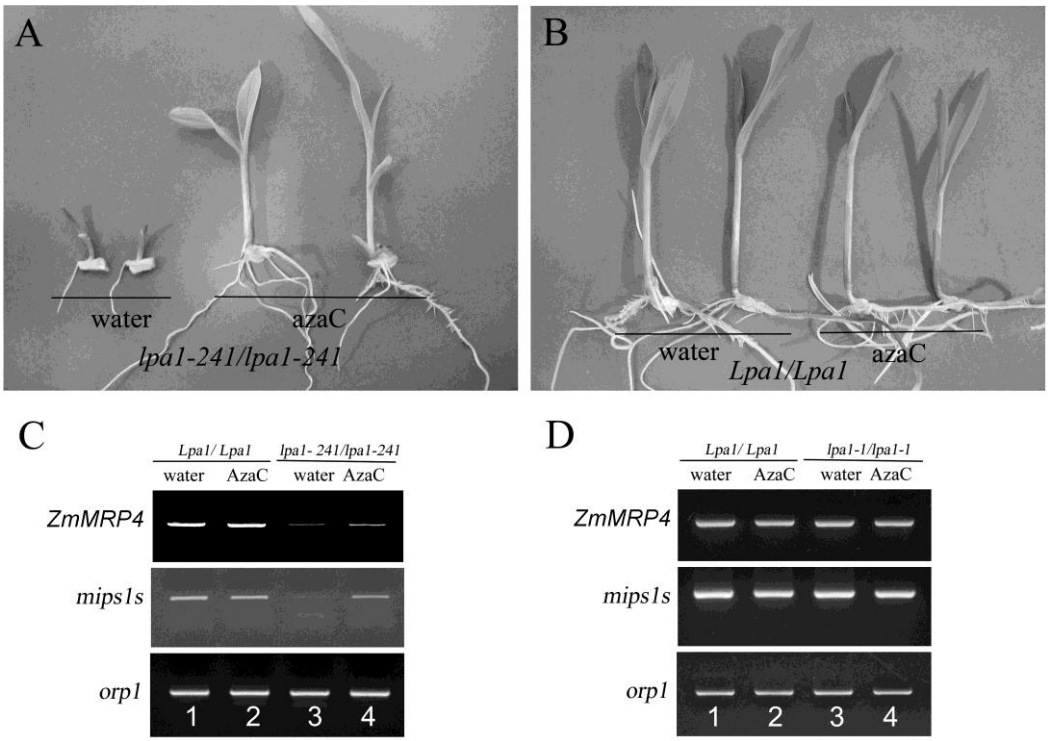

Figure 8. Effects of seeds 5-Azacytidine treatment on *lpa1-241* seedlings growth in vitro and on *mips1s* and *ZmMRP4* genes expression.
(A) 14 days old homozygous *lpa1-241* seedlings untreated (water) and treated (AzaC). (B) 6 days old *Lpa1/Lpa1* B73 seedlings, untreated (water) and treated (AzaC). Plants were obtained by embryos removed aseptically from the seeds previously treated and transferred to Murashige and Skoog tissue culture medium. (C) RT-PCR analysis showing the expression of *ZmMRP4* and *mips1s* gene in wild type (untreated, lane 1; treated, lane 2) and homozygous *lpa1-241* (untreated, lane 3; treated, lane 4). *Orp1* gene amplification is shown as control. (D) RT-PCR analysis of expression of *ZmMRP4* and *mips1s* gene in wild type (untreated, lane 1; treated, lane 2) compared to homozygous *lpa1-1* (untreated, lane 3; treated, lane 4). *Orp1* gene amplification is also shown.

Table 1. Effects of generation on heritability of HIP phenotype in *lpa1-241* lines. The seeds obtained were assayed for free P using a microtiter plate-based colorimetric assay (Chen *et al*. 1956) and visually classified as wild type (Wt), weak (W), intermediate (I) and strong (S).

cross	generation	Phenotypic classification			
		Wt	W	I	S
Lpa1$_{B73}$*/lpa1-241* selfed	F_2	45 (31.9 %)	57 (40.4 %)	8 (5.7%)	31 (22 %)
Lpa1'$_{B73}$*/lpa1-241* selfed	F_3	39 (24.1%)	24 (14.8%)	39 (24.1)	60 (37%)
Lpa1''$_{B73}$*/lpa1-241* selfed	F_4	2 (1.9%)	21 (19.6%)	65 (60.7%)	19 (17.8%)

Table 2. Effects of generation on heritability of HIP phenotype in *lpa1-241* lines. *Lpa1* $_{B73}$*/lpa1-241* were crossed to *lpa1-1/lpa1-1* plants. The seeds obtained were assayed for free P using a microtiter plate-based colorimetric assay (Chen *et al.* 1956) and visually classified as wild type (Wt), weak (W), intermediate (I) and strong (S).

cross	generation	Phenotypic classification			
		Wt	W	I	S
Lpa1 $_{B73}$*/lpa1-241* X *lpa1-1/lpa1-1*	F_1	65 (30.5%)	40 (18.8%)	8 (3.7%)	100 (46.9%)
Lpa1' $_{B73}$*/lpa1-241* X *lpa1-1/lpa1-1*	F_2	45 (24.9%)	29 (15.9%)	20 (10.8%)	90 (48.6%)
Lpa1'' $_{B73}$*/lpa1-241* X *lpa1-1/lpa1-1*	F_3	9 (9.4%)	22 (22.9%)	21 (21.9%)	44 (45.8%)

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ISOLATION OF A MAIZE *low phytic acid 1* ALLELE

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Introduction

Phytic acid, myo-inositol 1,2,3,4,5,6-hexakisphosphate (IP6), is the main accumulation form of phosphorous in plants, it is accumulated in pollen and seeds (up to 4-5% of dry weight). In maize kernel the 20% of phytic acid (PA) is accumulated in the aleuronic layer while the remaining 80% in the scutellum. During germination phytic acid is hydrolyzed by phytases. Phytic acid and the cations that it is able to bond are poorly bio-available for monogastric animals due to their lack of phytase activity. One approach to solve this problem is the isolation of cereal mutants that accumulate low level of phytic P in the seeds. The *low phytic acid* (*lpa*) mutations exhibited not only a reduction of PA in the seed, but also a proportionally augmentation of free inorganic P without variation in the total phosphorous content. (Pilu *et al.* 2005, Raboy 2009)

Key Words: maize, low phytic acid, mutant.

Material and Method

Mutagenic treatment and mutants isolation

A population of EMS(ethylmethane-sulfonate)-induced mutants was generated from the K6 stock using the seed-treatment method (Neuffer 1994). The treated seeds were planted and the plants obtained self-pollinated, obtaining 300 M2 ears. The screening by the molybdate staining assay was able to recognize the mutant HIP (high inorganic phosphate) phenotype.

Assay for high phosphate levels in maize kernels

The extraction were performed on 100 mg of the flour adding 1 ml 0.4M HCl, incubating at room temperature for 1 h, to an aliquot of 100 µl of sample were added 900 µl 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. After 1 h of

incubation at room temperature a blue coloured phosphomolybdate complex arose if free phosphate was present (Chen *et al.* 1956).

A non-destructive assay for HIP phenotype were performed in order to maintain the viability of the seeds. The scutellum was incised using a small milling cutter mounted on an electric drill. After that the flour obtained was placed in microtiter with 200 µl 0.4M HCl for 1 h at room temperature, then 800 µl of Chen's reagent. After 1 h HIP phenotype was recognized from the blue colour by visual inspection.

Embryo rescue

Seeds were sterilized with 5% (v/v) sodium hypochlorite for 15 min, and then incubated in sterile distilled water in rotating flasks at 30°C for 18 h. Embryos were aseptically removed and transferred to Murashige and Skoog salt mixture (pH 5.6) containing 2% (w/v) sucrose, solidified with 0.8% (w/v) agar (Phytagel™). We incubated the cultures in a growth chamber at 25°C with a 14/10 light/dark photoperiod (Pilu *et al.* 2005).

Results and Discussion

In the present work, following chemical mutagenesis, we isolated a recessive maize mutant, named provisionally *lpa1-**, with relevant increase in grain-free phosphate content.

We obtained a mutant population by the EMS treatment and 300 M2 families were screened using the molybdate staining method for free phosphate.

We found a *low phytic acid 1* mutant (provisionally named *lpa1-**) that causes an increase in the amount of free phosphate; the 3:1 segregation ratio of *lpa1-**, observed in the F2 generation, indicated a monogenic recessive defect (Table 1).

The relationship of our low phytic acid mutation with the previously isolated low phytic acid maize mutations was tested. The mutants *lpa1-1*, *lpa1-241* and *lpa1-** were crossed in all combinations, their complementation results showed that the *lpa1-1* and *lpa1-241* mutant failed to complement *lpa1-**, suggesting its allelic nature (Table 2).

The *lpa1*-* mutants as homozygous are affected by negative side effects; we observed that the *lpa1*-* mutation in homozygous condition is lethal, *lpa1*-*/ *lpa1*-* seeds are not able to germinate in experimental and field condition.

This is not surprising since the IP6 plays a central role in important plant biological processes such as the synthesis of carbohydrates belonging to the raffinose family, cell-wall components, phosphorilated compound involved in membranes biogenesis, it is also involved in chromatin remodeling, in the editing of RNA, in the DNA repair and control of gene expression (Pilu *et al.* 2005, Pilu *et al.* 2009, Raboy 2009).

Strong pleiotropic effect of the *lpa1* class of mutants were also reported by previous studies (Pilu *et. al* 2005, Pilu *et. al* 2009).

The embryos obtained from *lpa1*-*/ *lpa1*-* HIP mature seeds cultured in MS medium grew slower than the wild type and some defective seedlings were observed. By visual inspection of the in-vitro cultivated embryos we observed that mutant embryos displayed a reduction in dimension and alteration in the shoot and in the root primordia. The in-vitro cultivation experiments indicate that germination could be partially restored by embryo-rescue and that by this mean it is possible to obtain viable plant (Figure 1).

Genetic analysis of this mutation, as well as its biochemical characterization are under way.

Acknowledgments

We wish to thank Dr Victor Raboy, USDA ARS,Aberdeen, ID, USA, for his generous gift *of lpa1-1* seeds and Dr. Andrea Bucci for his hard work in the field.

Figures and Tables

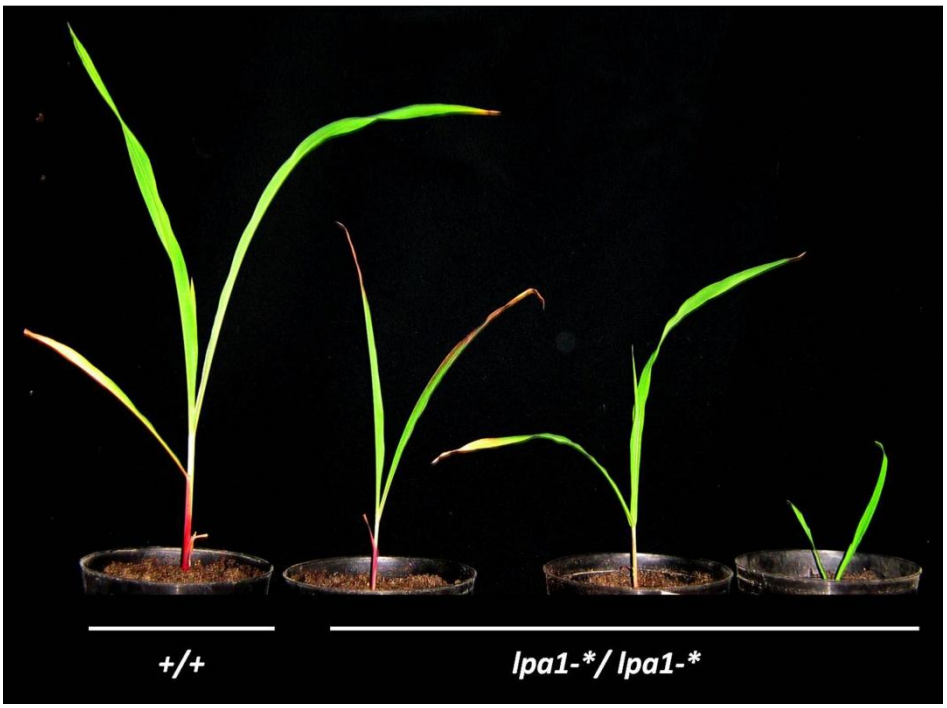

Figure 1: Effect of *lpa1-** mutation in homozygous condition on plant development. The *lpa1-*/ lpa1-** plants were obtained by embryo rescue treatment.

Table 1. Segregation of +/*lpa1-** phenotypes observed in the F2 progenies obtained by selfing.

	Genetic test	Segregation		χ^2	*p*
		wt	mutant		
+/*lpa1-**	F2	159	51	0.057	0.8113

Table 2. Complementation test among *lpa1-1*, *lpa1-241* and *lpa1-**

	lpa1-1	*lpa1-241*	*lpa1-**
lpa1-1	-	-	-
lpa1-241		-	-
*lpa1-**			-

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STUDY OF *low phytic acid 1-7* (*lpa1-7*), A NEW *ZmMRP4* MUTATION IN MAIZE

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Abstract

Phytic acid, 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 hydrolized by phytases. When seeds are used as food/feed, phytic acid and the bound cations are poorly bio-available for human and monogastric livestock due to their lack of phytase activity. Reducing the amount of of phytic acid 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* 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 to 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: maize, low phytic acid, mutagenesis, multi-drug-resistance (MRP) protein, inositol phosphates, ATP-binding cassette (ABC) transporter.

Introduction

Phytic acid (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 phytic acid (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 forming mixed salts with mineral cations is mainly 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 undigested phosphorous contained in excreted phytin can contribute to water pollution (eutrophication) (Raboy 2009).

These negative effects have led to breeding programmes which 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 three 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 (Ins(3)P1) to PA synthesis) and mutations affecting the transport of phytic acid to the vacuole (Raboy 2009; Panzeri *et al.* 2011) (Figure 1).

In several crops *low phytic acid* 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 (Guttieri *et al.* 2004), in common bean by chemical mutagenesis (Campion *et al.* 2009), in rice by physical and chemical mutagenesis (Larson *et al.* 2000; Liu *et al.* 2007).

In maize three *low phytic acid* mutants have been isolated: *lpa1* (Raboy *et al.* 2000; Pilu *et al.* 2003) and *lpa2* (Raboy *et al.* 2000) by chemical mutagenesis, *lpa3* by transposon tagging (Shi *et al.* 2005) (Table 1).

Compared to 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-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 phytic acid accumulation and viability of seeds. *low phytic acid* 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 (Meis *et al.* 2003; Pilu *et al.* 2005; Bregitzer and

Raboy 2006; Guttieri *et al.* 2006; 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 *lpa1* locus and indicated the involvement of an epigenetic event of paramutation in the genetics of this trait (Pilu *et al.* 2009; Raboy 2009).

In this work we report data regarding the isolation and the characterization, under different aspects, of a new *low phytic acid* 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 (ethylmethane-sulfonate)-induced mutants (about 300 M2 ears) using the seed-treatment method (Neuffer 1994). We screened this population by the molybdate staining assay which was able to recognize the HIP phenotype (see Assay for Seed Free Phosphate Content). The newly isolated mutant was crossed with the two *low phytic acid 1* mutants (*lpa1-1* and *lpa1-241*) in all pair-wise 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 0.4M HCl. 100 µl of extract were 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 (Chen *et al.* 1956). After incubation of 1 h at room temperature we observed the blue coloured phosphomolybdate complex whose colour 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 KH_2PO_4.

In order to preserve the growth capacity of the seed we performed a non-destructive 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 0.4M 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 surnatant 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 1h 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 phytic acid dodecasodium salt (Sigma, product n° P-8810, St Louis, MO, USA).

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 colour 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).

Assay for seed total P content

For the determination of total P we added to 100 mg of flour samples 10 ml of 65% HNO_3 in Teflon tubes, then we digested the sample by a microwave digestor system (Anton Paar MULTIWAVE 3000) in Teflon tubes by applying a two step power ramp (step 1: at 400W in 5 min maintained for 10 min; step 2: at 1000W 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 (^{6}LI, ^{45}Sc ^{89}Y) to both samples and calibration curve to give a final concentration of 20µg 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; Sigma, product n° M5519, St Louis, MO, USA) containing 2% (w/v) sucrose, solidified with 0.8% (w/v) agar (Phytagel™, Sigma, product n° P8169, St Louis, MO, USA).

We incubated the cultures in a growth chamber at 25°C with a 14/10 light/dark photoperiod. The light source consisted of four 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 µmol m^{-2} s^{-1}.

5-Aza-2′-Deoxycytidine (Azacytidine) treatment

We sterilized mature dry seeds with 5% (v/v) sodium hypochlorite for 15 min, we incubated the seeds in 20 ml of 30 µM 5-aza-2′-deoxycytidine (Sigma, product n° A3656, St Louis, Mo, USA) 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 regulators treatments

We removed embryos aseptically as previously described and transferred them onto the Murashige and Skoog salt mixture medium (pH 5.6; Sigma, St Louis, MO, USA, product no. M5519) containing 2% (w/v) sucrose, solidified with 0.8% (w/v) agar (Phytagel™, Sigma, product n° P8169, St Louis, MO, USA). We supplemented the medium with the hormones IAA or GA, each at a concentration of 10 µM. 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 µm, 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 stereoscope 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 *lpa1-1* allele in *low phytic acid* mutants.

The allele-specific forward primers were designed on a single nucleotide substitution polymorphism in the *ZmMRP4* 10th exon (Shi *et al.* 2007) (Figure 9).

The *Lpa1* wild type specific forward primer was ZmMRP30L (5'-GTACTCGATGAGGCGACAGC-3'), whereas *lpa1-1* mutation specific forward primer was ZmMRP432L (5'-GTACTCGATGAGGCGACAGTG-3').

The reaction mix of the wild type *Lpa1* allele-specific amplifications contained an aliquot of genomic DNA, 1X Green Go Taq buffer (Promega, Madison, WI, USA), 2.5 µM $MgCl_2$, 0.2 µM each of dATP, dCTP, dGTP and dTTP, 0.3 µM of forward ZmMRP30L-specific primer, 0.3 µM of reverse ZmMRP410R primer (5'-CCTCTCTATATACAGCTCGAC-3') and 1.25 unit of Go Taq Flexy DNA polymerase (Promega, Madison, WI, USA), in a final volume of 25 µl. 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 *Lpa1* allele-specific amplification product was 468 bp long.

The reaction mix of the *lpa1-1* allele-specific amplifications was identical to that of wild type *Lpa1* allele-specific amplifications, except that 0.3 µM of ZmMRP32L *lpa1-1* specific primers were used instead of ZmMRP30L. 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 *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 RT-PCR to detect *ZmMRP4* gene transcripts. We synthesized first strand cDNA using an oligo (dT) primer from total RNA. We performed on all RNA samples a DNase (1U μg^{-1}) treatment before cDNA synthesis. We used the first strand cDNA as template for PCR, amplification reactions contained an aliquot of cDNA, 1X Green Go Taq buffer (Promega, Madison, WI, USA), 2.5 µM $MgCl_2$, 0.2 µM each of dATP, dCTP, dGTP and dTTP, 0.3 µM of each primer and 1.25U 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. We standardized the concentration of the different samples 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'-CTTCTCTATATACAGCTCGAC-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 *low phytic acid* (*lpa*) mutants we performed a seed mutagenesis treatment with EMS (ethyl-methanesulfonate) (Neuffer *et al.* 1978). Since wild type mature maize seeds contain high amount of phytic phosphate and low free phosphate content, we screened the mutagenized population looking for seeds containing high levels of free phosphate (HIP phenotype), a typical feature of *lpa* mutants (Rasmussen and Hatzack 1998; Raboy *et al.* 2000; Pilu *et al.* 2003).

We screened approximately 300 M_2 families by the assay of free phosphate on flour (as described in material and methods) and from an ear segregating for the HIP phenotype, we isolated one putative mutation that we provisionally named *lpa*-7.*

Preliminary data coming from the characterization of the *lpa*-7* indicated a strong reduction of the PA content in the kernel, compatible with a mutation belonging to the *low phytic acid 1* class (Table 1).

With the aim of verifying this hypothesis, we performed the allelism test with two *low phytic acid 1* mutants (*lpa1-1* and *lpa1-241*) isolated to date. Our mutant failed to complement the *lpa1* mutants so as to suggest an allelic relationship; so we renamed the new mutation *lpa1-7* (Table 2).

To confirmed 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* sequence-specific amplification polymorphism (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 (Figure 2).

The genetic analysis of *lpa1-7* was based on the detection of the presence/absence of the HIP phenotype. Assays conducted on kernels originated from segregant ears confirmed the correlation between the low phytic acid and HIP phenotypes.

The 3:1 segregation ratio of the *lpa1-7* mutant, observed in the M_2 family and successive heterozygous family, implies a monogenic recessive defect (Table 3). To confirm this finding, we selfed the M_2 family and the analysis on M_3 generation again exhibited the 3:1 segregation ratio, we observed the same ratio also in the M_4 generation (Figure 3).

We outcrossed to inbred line B73 the M_4 generation of *lpa1-7* and we observed in the F_4BC_1 seeds normal free inorganic P level in colorimetric assays. The 3:1 segregation ratio of the mutation arose again in the F_2 generation (Figure 3).

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 (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 ten-fold increase in the amount of free phosphate (Figure 4A) and a reduction of about 80 % of phytic acid compared to the wild type control (Figure 4B), without a significant alteration in the total P amount (Figure 4C).

lpa1-7 origin: gene silencing vs sequence mutation

It is known that *Lpa1* locus undergoes spontaneous silencing with high frequency generating epialleles such as *lpa1-241* (Pilu *et al.* 2009) and other *lpa1* alleles isolated in the past (Raboy, personal communication) with an anomalous inheritance for a recessive trait. With the aim of establishing the origin of our new mutant, we considered the penetrance and the expressivity of *lpa1-7* mutation: as shown in Table 3, the penetrance of *lpa1-7* mutation was of 100% (χ^2 = 0.13 with

the expected segregation value for the HIP phenotype of 3:1) and the expressivity was higher with respect to the *lpa1-241* allele (data not showed). To strengthen these results we performed reverse transcriptase-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 (Figure 5). 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 (Pilu *et al.* 2009). Neither treated wild-type seeds nor homozygous *lpa1-7* seeds revealed significant differences compared to untreated controls (data not shown).

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 partially restore lacking germination. The wild-type siblings germinated and grew regularly, while only a fraction of mutants generated seedlings: they were characterized by slow growth and abnormal morphology in particular at the level of the root apparatus (Figure 6A, 6B). 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 6C). 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 6E) compared to the wild type (Figure 6D), 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 6E); 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 6F, 6G).

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 6B). 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 6H, 6I). 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 7C). 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 6M) compared to wild siblings control (Figure 6L) as confirmed by measurements on the captured images (Figure 7A, 7B).

Discussion

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 low phytic acid character and the study of the PA biological pathway represent an objective for crop genetic improvement programmes (Raboy 2009).

So far in maize three *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) (Table 1). 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 subsequently genetic studies on the HIP phenotype identification.

In this work, we present data regarding a *low phytic acid* mutant obtained by chemical mutagenesis, we isolated a recessive *low phytic acid* maize mutant, named provisionally *lpa*-7*, exhibiting a composition in P fraction in the kernel (Figure 4) compatible with a mutation of *lpa1* class (Table 1). In order to ascertain this point we performed the allelism test with two other *lpa1* mutants: *lpa1-1* (Raboy *et al.* 2000) and *lpa1-241* (Pilu *et al.* 2003). Our mutant failed to complement these mutants (Table 2), and data collected from genetic (Figure 3, Table 3) and molecular (Figure 2) analysis indicated the inheritance of *lpa1-7* as a recessive monogenic mutation, thus we renamed the new *low phytic acid 1* allele as *lpa1-7*. Since it is known that maize *lpa1* alleles can be affected by epigenetic events at a relatively high rate (Pilu *et al.* 2009; Raboy 2009) we conducted two experiments to ascertain the nature of *lpa1-7* 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. In fact previous work demonstrated that a phenomenon of gene silencing is involved in *lpa1-241*'s origin, causing a reduction in *ZmMRP4* expression, a non mendelian inheritance of this character and furthermore 5-Azacytidine treatment (demethylating agent) reverted the *ZmMRP4* gene expression and enabled recovery from the pleiotropic effects (Pilu *et al.* 2009). The gene expression analysis of *ZmMRP4* conducted on *lpa1-7* mutant did not reveal significant variations between the mutant and the wild type (Figure 5) and the 5-Azacytidine treatment did not show differences compared to untreated controls (data not shown) indicating that the molecular lesion due to *lpa1-7* mutation did not affect the gene transcription but is likely to be caused by a sequence mutation in *ZmMRP4* coding sequence as found also for the *lpa1-1*

mutation (Pilu *et al.* 2009). Thus *lpa1-1*, and *lpa1-7* did not show differences in *ZmMRP4* expression in contrast with the *lpa1-241, lpa1-mum* knockout mutant where the transposon insertion in the gene stopped its expression (Shi *et al.* 2007) and *lpa2* mutation in rice (Xu *et al.* 2009) where the mutants showed a lower expression level. With the aim to individuate the sequence lesion in the *ZmMRP4* gene the sequencing of the *lpa1-7* coding sequence is in progress.

The homozygous *lpa1-7*/*lpa1-7* were not able to germinate probably because the mutation causes approximately a ten-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 4). These data are in agreement with previous studies indicating a limit for PA reduction compatible with seed viability of about 55–65%, in fact the viable *lpa1-1* mutant has a reduction of PA of about 65% (Raboy *et al.* 2001) whilst all the not viable mutants exhibiting a higher reduction in PA such as *lpa1-241* (Pilu *et al.* 2003) and *lpa1-mum* (Shi *et al.* 2007) have a PA reduction of about 90 %. These observations emphasize the important role of MRP transporter encoded by *Lpa1* gene, a null allele of *ZmMRP4* is lethal as homozygous and a copy, also intermediate or hypomorphic, is fundamental for seed viability. Studies conducted on rice *lpa2* mutant, homologous orthologous of the maize *lpa1* mutant, showed the same behavior (Xu *et al.* 2009).

The lethality of *lpa1-7* 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 6A, 6B). 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 *lpa1-1* 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 *lpa1-7* embryos were smaller than wild type controls (Figure 6D, 6E).

We noticed alterations in the root primary meristem of the mutant embryo: the ground meristem and the procambium (between the root apical meristem and the shoot primordia) exhibited less organized stuctures (Figure 6F, 6G). 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 (ROS) 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 while 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 *Pvmrp1 MRP* transporter, homologous to *ZmMRP4*, is able to confer the low phytic acid 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, *PvMrp2*, 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 *low phytic acid* mutations in several crops (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.* 1997) (Figure 6H, 6I) and a decrease in

trichomes length coming with an increased trichome density on leaf margins (Figure 7). 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 three distinct types of trichomes, the bigger are the macrohairs that are considered as a marker for adult leaf identity and are regulated from *macrohairless1* (*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* non epigenetic 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 *low phytic acid* 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.

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Figures and Tables

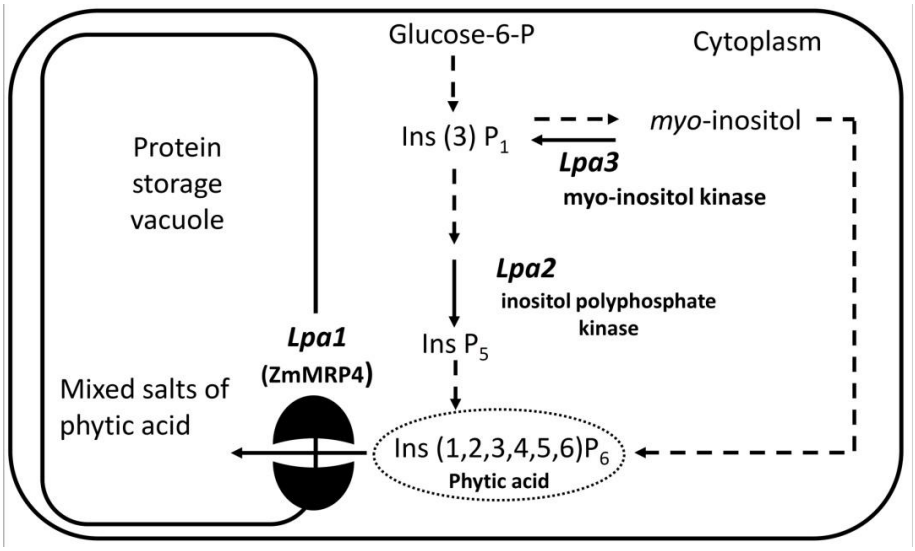

Figure 1. Schematic model of the biosynthetic pathways of phytic acid accumulation in maize seed. The three low phytic acid mutations so far isolated in maize are shown: starting from the activity of Myo–Ins(3)P1 synthase, which converts glucose-6-P to Ins(3)P1 that can be produced also by a myo-Ins kinase activities encoded by *Lpa3* gene. *Lpa2* encodes an Ins phosphate kinase while *Lpa1* gene (*ZmMRP4*) encoded for the phytate transporter involved in the compartmentalization into protein storage vacuoles (adapted from Pilu *et al.* 2009).

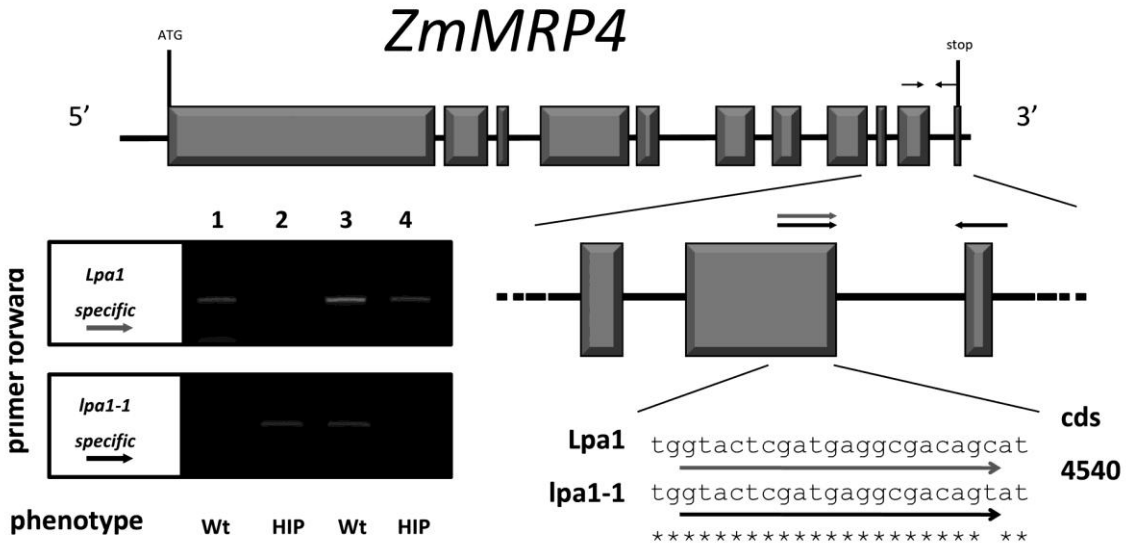

Figure 2. Genetic analysis based on the S-SAP marker. Diagram showing exon-intron structure of *ZmMRP4* gene, the position of the primers and the alignment between the *Lpa1* and *lpa1-1* sequence. Shown are the amplification patterns of *Lpa1/Lpa1* (lane 1), *Lpa1/lpa1-1* (lane 2), *lpa1-1 /lpa1-1* (lane 3) and *lpa1-7/ lpa1-7* (lane 4); their phenotype is also annotated.

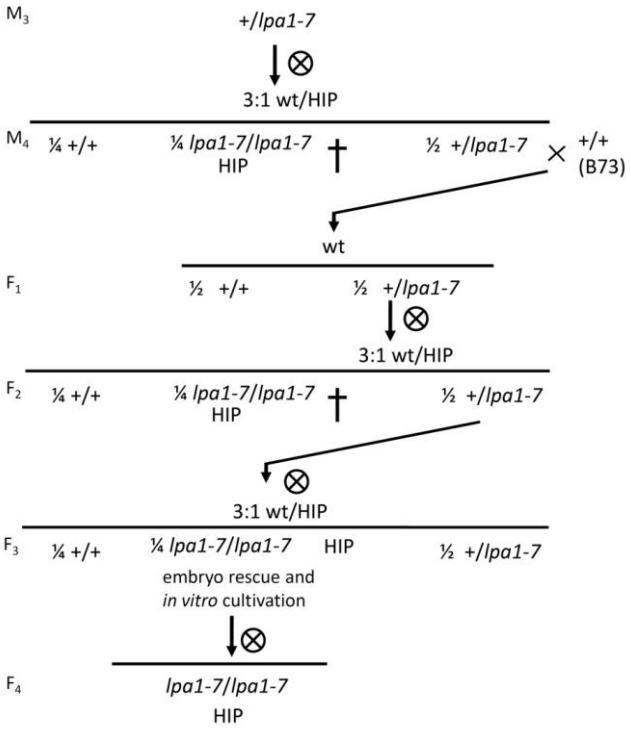

Figure 3. Pedigree diagram. Genetic analysis showed the inheritance of the *lpa1-7*, a monogenic recessive mutation lethal in homozygous condition († = lethal).

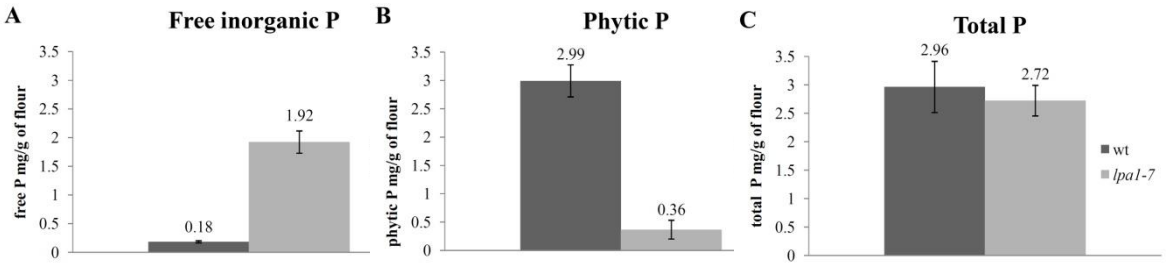

Figure 4. Mature dry seeds were assayed for free inorganic P (A), phytic acid P (B) and total P (C). The P fractions were expressed as mg of P (atomic weight = 31) on gr of flour. Confidence intervals at 95% are shown.

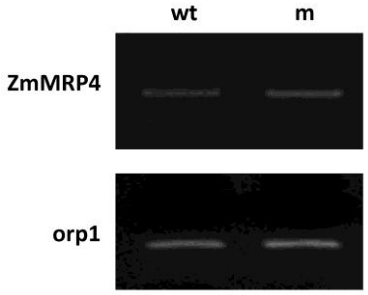

Figure 5. Reverse transcriptase-PCR (RT-PCR) analysis showing the expression of *ZmMRP4* gene in wild type (lane 1) and *lpa1-7* (lane 2). *orp1* gene amplification is shown as control.

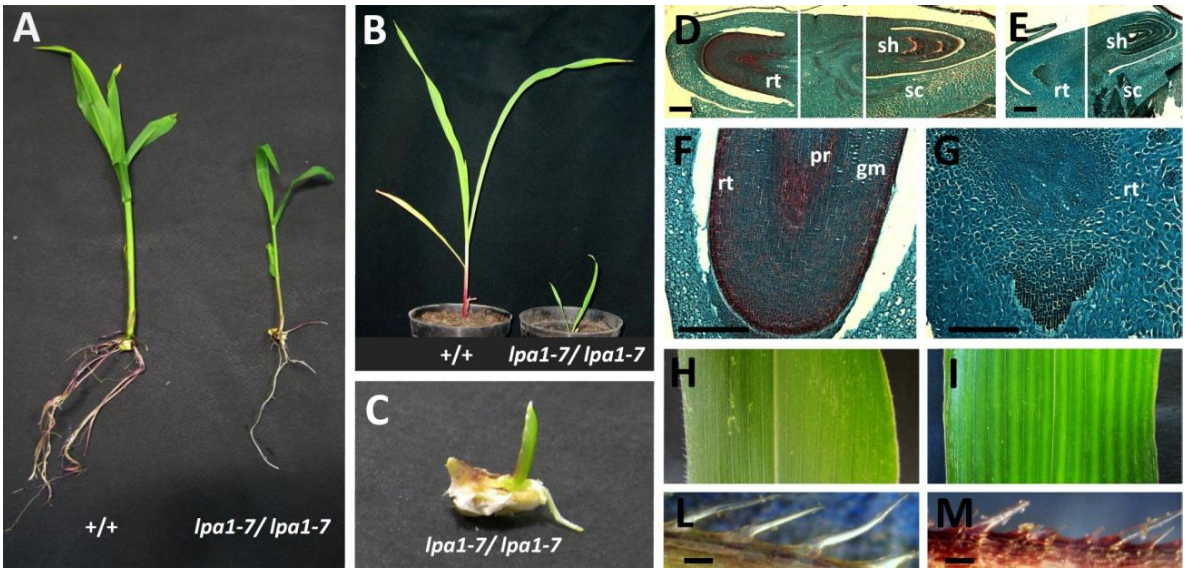

Figure 6. Negative pleiotropic effects of *lpa1-7* on seedling growth in vitro (A) and on plants (B). The mutation causes a defective primary root in seedling (C). Longitudinal sections of mature wild-type (D) and *lpa1-7* (E) kernels. Magnification of wild-type (F) and *lpa1-7* (G) root primordia. The sections were stained with safranine-fast green. (gm, ground meristem; sc, scutellum; sh, shoot; rt, root; pc, 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.

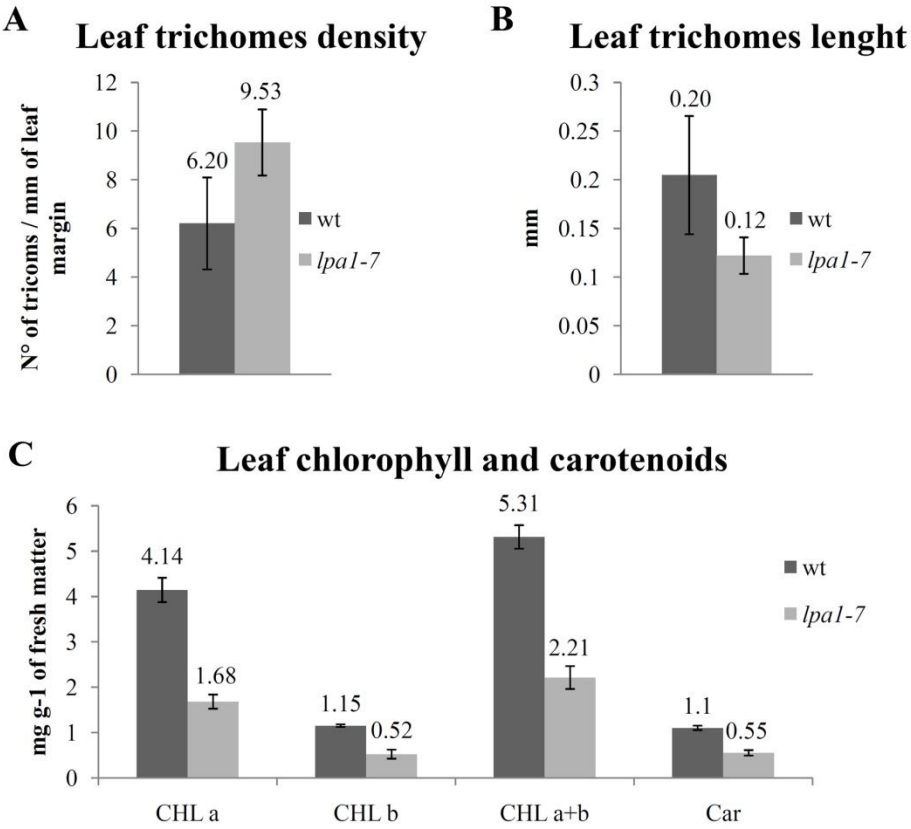

Figure 7. Determination of trichomes' length (A) and density (B). Determination of: chlorophylls a, b, a+c and carotenoids content (C). We performed the analysis on mature apical leaves. Confidence intervals at 95% are shown.

Table 1. *low phytic acid* mutants isolated in maize and their effect on seed phenotype.

Mutant	Function	Reduction in phytic acid	Free P level	Total P content	Intermediate accumulated	Ref.
lpa1	ZmMRP4 ABC transporter	-60% to -95%	High	Unaffected	None	Raboy *et al.* (2000)
lpa2	Ins(1,3,4)P_3 5/6 kinase	-50%	High	Unaffected	Inositol phosphate	Raboy *et al.* (2000)
lpa3	*Myo*-inositolo kinase	-50%	High	Unaffected	*Myo*-inositol	Shi *et al.* (2005)

Table 2. Allelism test among *lpa1-1*, *lpa1-241* and *lpa*-7*.

	lpa1-1	*lpa1-241*	*lpa*-7*
lpa1-1	-	-	-
lpa1-241		-	-
lpa-7*			-

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THE *low phytic acid1-241* (*lpa1-241*) MAIZE MUTATION ALTERS THE ACCUMULATION OF ANTHOCYANIN PIGMENT IN THE KERNEL

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Abstract

The *lpa1* mutations in maize are caused by lesions in the *ZmMRP4* (*multidrug resistance-associated proteins* 4) gene. In previous studies (Raboy *et al.* 2000; Pilu *et al.* 2003a; Shi *et al.* 2007), several mutations have been isolated in this locus causing a reduction of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, or $InsP_6$) content and an equivalent increasing of free phosphate. In particular the *lpa1-241* mutation causes a reduction of up to 90% of phytic acid, associated with strong pleiotropic effects on the whole plant. In this work, we show, for the first time to our knowledge, an interaction between the accumulation of anthocyanin pigments in the kernel and the lpa mutations. In fact the *lpa1-241* mutant accumulates a higher level of anthocyanins compared to wild type either in the embryo (about 3.8 fold) or in the aleurone layer (about 0.3 fold) in a genotype able to accumulate anthocyanin. Furthermore we demonstrate that these pigments are mislocalized in the cytoplasm, conferring a blue pigmentation of the scutellum, because of the neutral/basic pH of this cellular compartment. As a matter of fact the propionate treatment, causing a specific acidification of the cytoplasm, restored the red pigmentation of the scutellum in the mutant and expression analysis showed a reduction of *ZmMRP3* anthocyanins' transporter gene expression. On the whole these data strongly suggest a possible interaction between the *lpa* mutation and anthocyanin accumulation and compartmentalization in the kernel.

Key Words: anthocyanin transporter, *Colored1* gene, *lpa* mutation, maize

Abbreviations: HIP High inorganic phosphate, InsP *myo*-inositol phosphates, Lpa Low phytic acid

Introduction

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phospate, or $InsP_6$) is the most plentiful form of phosphate present in cereal kernels as well as in seeds of most plants (O'Dell *et al.* 1972; Raboy *et al.* 1990). Phytic acid is accumulated in the seed (in corn mainly in the scutellum while in rice, barley and wheat in the aleurone layer), as a mixture of phytate salts of several cations such as potassium, iron, zinc, magnesium, etc (Raboy 2002). During seed germination, this molecule is degraded by phytase activity releasing free phosphate, *myo*-inositol and cations, necessary for seedling growth. Monagastric animals are not able to digest and thus utilize phytate salts that exhibit a further anti-nutritional activity in the feed interfering also with protein and starch digestion. For these reasons several breeding programmes aiming to develop cereals and legumes with lower levels of phytic acid compared to traditional cultivars are underway. *lpa* mutants have been isolated in maize by chemical mutagenesis (Raboy *et al.* 2000; Pilu *et al.* 2003a) and by transposon tagging (Shi *et al.* 2005), in barley by chemical mutagenesis (Larson *et al.* 1998; Rasmussen and Hatzack 1998; Bregitzer and Raboy 2006), in wheat by chemical mutagenesis (Guttieri *et al.* 2004), in rice by chemical and physical mutagenesis (Larson *et al.* 2000; Liu *et al.* 2007), in soybean by chemical mutagenesis (Wilcox *et al.* 2000; Hitz *et al.* 2002) and physical mutagenesis (Yuan *et al.* 2007) and in common bean by chemical mutagenesis (Campion *et al.* 2009).

However phytic acid, by firmly chelating iron cations, is able to oppose the formation of reactive oxygen species (Graf *et al.* 1984) and is thus involved in the preservation of viability of plant seeds as conjectured by Graf and Eaton (1990) and Doria *et al.* (2009).

So far among of the three *lpa* mutations isolated in maize (*lpa1*, *2* and *3*), involved in the phytic acid biosynthesis, *lpa1* showed the lowest phytic acid content in the seed (Raboy *et al.* 2000; Pilu *et al.* 2003a). This mutation does not modify the total amount of seed P but reduces phytic acid content thus leading to a proportionally increased level of free phosphate (Raboy *et al.* 2000; Pilu *et al.* 2003a; Shi *et al.* 2005). Owing to this, an HIP (high inorganic phosphate) phenotype, is diagnostic for the presence of *lpa* mutant seeds. Transposon mutagenesis experiments conducted by

Shi *et al.* (2007) demonstrated that *lpa1* gene encodes a multidrug-associated-protein (MRP) named *ZmMRP4* (accession number EF586878). MRP proteins are transmembrane transporters involved in several functions such as organic ions transport, xenobiotic detoxification, oxidative stress tolerance and transpiration control (Swarbreck *et al.* 2003; Klein *et al.* 2006).

In a previous work (Pilu *et al.* 2003a) we have isolated and characterized an *lpa* mutation named *lpa1-241* allelic to *lpa1-1*, the first mutation isolated by Raboy (2000). Unlike the *lpa1-1* mutation that is stable, being caused by a molecular lesion in the *ZmMRP4* sequence (Shi *et al.* 2007), *lpa1-241* mutation shows a variable expression and genetic data suggest that an epigenetic phenomenon might be involved in this trait (Pilu *et al.* 2009). Moreover, *lpa1-241* and other *lpa1* strong mutations, showed several negative pleiotropic effects, in particular lack of germination in seeds having less than 20% of phytate amount compared to wild type (Raboy *et al.* 2001; Pilu *et al.* 2005).

In this work, with the aim to study a possible interaction between phytic acid accumulation and anthocyanin biosynthesis, we produced lines carrying *lpa1* mutations and the genes involved in the anthocyanin biosynthesis.

Anthocyanin are water soluble secondary metabolites belonging to the class of flavonoids, molecules synthesized in maize by a complex pathway made up of more than 20 genes, and regulated by two classes of transcription factors: *r1/b1* bHLH genes and *c1/pl1/p1* MYB gene families (Chandler *et al.* 1989; Dooner *et al.* 1991; Pilu *et al.* 2003b).

Anthocyanin are synthesized exclusively in plants having red coloured tissues in which these molecules are present in the glycosylated form inside the vacuole where their colour is partially dependent on the pH, ranging from dark red to bluish (de Vlaming *et al.* 1983). In plants these pigments play important roles in particular in the recruitment of pollinators, signaling with microbes, male fertility, antimicrobial activity, UV protection and in general they protect from oxidative damage (reviewed by Winkel-Shirley 2002). In maize, anthocyanin are cytoplasmically synthesized and transported in the vacuole probably by *ZmMRP3* gene product activity (Goodman *et al.* 2004). However, so far, this process is

poorly understood and ZmMRP3 does not seem to be the only protein involved in this process (Goodman *et al.* 2004).

In this study we observed that the *lpa1-241* mutation enhances the accumulation of anthocyanin in the kernel, changing the colour of scutellum in the *lpa1-241* strongest mutant from dark red to bluish. Furthermore, here we present genetic, physiological, histological and molecular data supporting the hypothesis that the observed change of anthocyanin colour is due to a defect in the pigment transport in the vacuole, causing a mislocalized accumulation of these pigments in the cytosol.

Materials and Methods

Genetic stocks

The *lpa1-241* mutant was originally isolated from the M_2 progeny of chemically (ethyl methane sulphonate, EMS) mutagenized populations (Pilu *et al.* 2003a). The *lpa1-1* mutant stock was kindly provided by Dr. Victor Raboy, USDA ARS, Aberdeen, ID, USA (Raboy *et al.* 2000).

Plants heterozygous for *lpa1-241* and *lpa1-1*, in the same background (B73 line) were used as donors in crosses with plants carrying *R-sc* allele of *R1* (*colored1*) gene (W22 line) and *Sn:bol3* gene to produce the NILs (near isogenic line) *R-sc/R-sc +/lpa1-241*, *R-sc/R-sc +/lpa1-1* and *Sn:bol3/ Sn:bol3 +/lpa1-241* used in this work. *R-sc*, a germinal derivative of the paramutagenic allele *R-st* (Kermicle 1984), confers coloured aleurone and scutellum while the plant is green. *Sn:bol3* is a locus lying two map units distal to the *R1* locus, conferring specific pigmentation after light exposure to the scutellar node, mesocotyl, leaf base and midrib (Pilu *et al.* 2003b).

Quantitative free phosphate assay (detection of HIP phenotype)

Seeds were ground in a mortar with a steel pestle and 100 mg of the resulting flour was extracted with 1 ml 0.4 M HCl for one hour at room temperature. Samples were mixed briefly and 100 µl were removed and supplemented with 900 µl

Chen's reagent (6 N H_2SO_4: 2.5% Ammonium Molybdate: 10% Ascorbic acid: H_2O 1:1:1:2, by vol.) in microtiter plates (Chen *et al.* 1956). To test the seeds for the presence of the *lpa1* mutation without interfering with their germination or growth ability, a small amount of seed flour (about 10 mg) was obtained from a single incision by a hand drill. The flour was extracted with 200 µl 0.4 M HCl and then 800 µl of Chen's reagent was added. After 1 h a strong HIP phenotype (≥ 1.4 P free mg mg^{-1}) could be detected by visual inspection as previously reported by Pilu *et al.* (2009).

Analysis of anthocyanin by spectrophotometer

Anthocyanins were extracted from flours (50 mg-100mg) using a 1% HCl in 95% ethanol extraction solution (2-3 ml) and quartz sand in a mortar (the flours from aleurone layer and embryo were obtained using a hand drill).

The extracts were centrifuged twice (7300 *g* for 15 min) and their absorption determined spectrophotometrically at 530 nm. The amount of anthocyanin was calculated as cyanidin 3-glucoside equivalents (molar extinction coefficient (□) 26900 L $m^{-1}mol^{-1}$, M.W. 484.82) for 100g of dry flours.

Extraction and HPLC analysis of anthocyanin

In order to identify the anthocyanin pigments, we performed HPLC analysis on *lpa1-241* and control kernels (four replicates each). Briefly, 20 ml of 2M HCl in methanol were added to 0.5 g of fine seed flour. This mixture was placed in a heated plate at 70/80 °C for 20 min to reduce the volume to about 2 ml. After centrifugation at 7300 *g* for 5 min, the surnatant was collected, cooled, filtered through a 0.2 µm nylon membrane and injected into a HPLC Kontron Instrument 420 system, equipped with C18 reverse phase Zorbax ODS column, 250 x 4.6 mm, 5 µm, Agilent Technologies. according to the method of Astadi *et al.* (2009), slightly modified. Mobile phase was: solvent A, 10% formic acid; solvent B: 100% acetonitrile. The gradient used was: from 0 to 8 min, A from 96% to 85%, B from 4% to 15%; from min 8 to 23, A 85%, B 15%. The injection volume was 20 µl and the flow rate was 1 ml/min. The detector wavelength was fixed at 530 nm.

Authentic cyanidine monoglucoside was dissolved in 1.2M HCl in methanol and used as standard.

Lpa1 allele molecular genotyping

DNA extraction was performed using flour or shoots as described by Dellaporta *et al.* (1983). Allele genotyping was performed by PCR amplification of a *ZmMRP4* sequenced gene portion (accession number EF586878). Allele-specific primers were designed on a two nucleotides insertion polymorphism found in the ACR *ZmMRP4* 10th intron. ACR specific primer was ZmMRP4+6092Ra (5'-AATCAAGACGATGAGAAAAGTTAT-3'), the B73 specific primer was ZmMRP4+6092Rb (5'-AATCAAGACGATGAGAAAAGTTC-3') and the forward primer was ZmMRP4+5590F primer (5'- TGGGAATGTGGTTTCTTAATGC -3'). The ACR allele specific amplification product is 498 bp long, the B73 allele specific amplification product is 503 bp long. Amplification products were visualized on 1.5% (w/v) agarose gels with ethidium bromide staining.

Cytosol acidification with propionate

Modification of cytosolic pH was performed using propionic acid, a cell permeant weak acid buffer as described by Kania *et al.* in 2003.
Whole embryos were excised from seeds (wt and *lpa1-241* homozygous) and imbibed in sterile deionized water for 18 h. The embryos were incubated at room temperature in Eppendorf tubes with 1 ml of 20 mM propionate Mes-KOH pH 4, or Mes-KOH pH 4 as a control (Kania *et al.* 2003). Images of treated embryos were taken after 2 h of incubation. Buffers were prepared with propionic acid ACS reagent (No. 402907, Sigma, St. Louis, MO, USA), Mes hydrate (No. M-8250, Sigma), Hepes (No. H3375, Sigma), and KOH (No. 221473, Sigma).

Vanadate treatment

For treating with vanadate, seeds (wt and *lpa1-241* homozygous) were first sterilized with 100% ethanol for 5 min and 5% sodium hypochlorite for 15 min, rinsed in sterile deionized water, and imbibed at 30°C for 48 h in rotating flasks

with 20 ml of liquid culture medium containing vanadate (1.6 g/l Hoagland's salt, 2 % sucrose, 5 mM Na_3VO_4). After imbibition, seeds were sterilized again for 2 min with 5% hypochlorite and rinsed in sterile deionised water, then the embryos were excised and incubated in a growth chamber at 25 °C with a 18/6 light/dark photoperiod (the light source consisted of four cool white F36T12/CW/HO fluorescent lamps from GTE Sylvania, Lighting Products Group, Danvers, MA, USA) in 20 ml culture tubes with 1 ml of liquid medium containing vanadate. Images of treated seedlings were taken after 48 h of growth. As control, a medium without vanadate was used (1.6 g/l Hoagland's salt, 2% sucrose). The vanadate medium was prepared from a 100 mM Na_3VO_4 stock solution in water, pH 6.5 adjusted with Mes (sodium vanadate, Sigma, No. S6508-10C; Mes hydrate, Sigma, No. M-8250) (Bogoslavsky and Neumann 1998).

Embryo rescue

Mature dry seeds were sterilized with 5% sodium hypochlorite for 15 min and then rinsed in sterile distilled water overnight. Embryos were removed aseptically and transferred to Murashige and Skoog salt mixture (pH 5.6) containing 2% sucrose, solidified with 0.8% agar (Plant agar, Duchefa, Haarlem, The Netherlands). Cultures were incubated in a growth chamber at 25°C with a 14/10 light/dark photoperiod and sampled for the following studies.

Histological analyses

For the light microscopy studies, *R-sc/R-sc lpa1-241/lpa1-241* mutant and *R-sc/R-sc* +/+ wild type kernels were imbibed in water for 18 h and the scutellum excised by scalpel and fixed in freshly prepared 100% ethanol: glacial acetic acid (3:1, v/v) at 4°C for 24 h. The fixed material was placed in 70% ethanol and stored at 4°C until processed. Following successive dehydration in an ethanol series and embedding in Paraplast Plus, 15-□m-thick sections were cut and serially arranged on microscope slides. To preserve anthocyanin pigments *in situ*, sections were mounted on slides covered with tert-butyl alcohol instead of water. Images and cell sizes were taken using a Zeiss IMAGE R.D1 microscope equipped with an

AxioCam MRc1 digital camera.

RT- PCR expression analysis

Total RNA was extracted from frozen whole embryos and shoots obtained from wt and *lpa1-241* homozygous using the method described by van Tunen *et al.* (1988). The scutellums were excised from seeds imbibed in sterile deionized water for 18 h and the shoots of 9-10 days old seedlings were obtained as previously described in "Embryo rescue". Reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect *ZmMRP4* and *ZmMRP3* gene transcripts. A set of primers specific for the *orange pericarp 1* (*orp1*) gene, which encodes the *β*-subunit of tryptophan synthase (Wright *et al.* 1992), was used to standardize the concentration of the different samples. *orp1* specific sequences were amplified using the following primers: The upstream primer, 5'-AAGGACGTGCACACCGC-3', and downstream primer, 5'-CAGATACAGAACAACAACTC-3'. The length of the amplified product was 207 bp. Several cycles of successive cDNA dilutions and *orp1* amplification were done in order to obtain similar amplification signals in the different samples. *ZmMRP4* mRNA detection was conducted with specific primers designed on *ZmMRP4* genomic sequence (EF586878): ZmMRP4+5135F (upstream primer 5'-TCATGGTGTAAGTTGTATGTTTC -3') and ZmMRP4+6206R (downstream primer 5'- CCTCTCTATATACAGCTCGAC -3'). A 677 bp amplificate is obtained after 33 cycles of denaturation at 94 °C for 45 s, annealing at 60° for 1 min, extension at 72° for 1.5 min.

ZmMRP3 mRNA detection was conducted with specific primers designed on *ZmMRP3* genomic sequence (AY609318): D63F (upstream primer 5'-GTGACGGGAAAGTAGTGGAGTA-3') and D64R (downstream primer 5'-CTGCCGCACAAGCATTTCTGT-3'). A 405 bp amplified product is obtained after 33 cycles of denaturation at 94 °C for 45 s, annealing at 65° for 1 min, extension at 72° for 1.5 min. Each expression analysis was conducted on RNA extracted from five individuals, in three replicates at least. PCR products were loaded on 2% (w/v) agarose gels and visualized by ethidium bromide staining under UV light.

Results

lpa1 mutations enhance the accumulation of anthocyanin pigments in the kernel

With the aim of constituting maize inbred lines carrying *lpa* mutations together with regulatory genes pushing the anthocyanin accumulation in the kernels and seedlings, heterozygous plants for *lpa1-241* and *lpa1-1* (B73 background) were used as donors in crosses with plants carrying *R-sc* allele (conferring kernel pigmentation in the aleurone and scutellum) and *Sn:bol3* (conferring seedling and plant pigmentation) genes in W22 background. After four cycles of selfing we produced the NILs *R-sc/R-sc +/lpa1-241*, *R-sc/R-sc lpa1-1/lpa1-1* and *Sn:bol3/Sn:bol3 +/lpa1-241* and the correspondent *R-sc/R-sc* +/+ and *Sn:bol3/Sn:bol3* +/+ NILs as controls. As shown in Figure 1 the level of anthocyanin in the *lpa1-241* homozygous whole kernel was significantly (Student's *t* test at $P \leq 0.05$) higher, by about 25 % , than in wild type control. This difference is mainly due to the higher anthocyanin content in the whole embryo of *lpa1-241* than of wild type (about 3.8 fold), while in the aleurone layer the *lpa1-241* content is higher only of about 0.3 fold.

The same phenomenon, although weaker (about an increase of 0.1 fold), was observed, in the case of *lpa1-1* kernels although the difference was not statistically significant (data not shown).

The presence of *lpa1-241* mutation causes a change of embryo coloration in the *R-sc* genotype

R-sc is an allele of *R-r* gene able to confer an accumulation of anthocyanin pigment in the aleurone and in whole embryo, in particular in the scutellum (Kermicle 1984). *Sn:bol3* drives instead the production of the same pigment in the germinated seedling. With the aim of rescuing the *R-sc/R-sc lpa1-241/ lpa1-241* seeds from segregating ears, after imbibition, whole embryos were excised from mature seeds and transferred to MS culture medium (see Material and methods). We noticed that the mutant embryos excised were bluish coloured, while the wild type

controls were red as expected for the presence of the *R-sc* allele (Figure 2A, B); furthermore respect to dry embryos both colours strongly increased their intensity after incubation on MS medium (in particular after seed imbibition for 18 h) indicating a *de novo* synthesis of anthocyanin in this tissue (data not shown). This coloration shift was not observed on seedling tissues in the *Sn:bol3/Sn:bol3 lpa1-241/lpa1-241* background (Figure 2C) indicating the tissue specificity behaviour of this phenomenon. Using the *lpa1-1* allele we did not observe any remarkable difference in the embryo colour of mutants in comparison with wild type (data not shown).

To confirm that the presence of *lpa1-241* mutation is the cause of this shift of coloration and of anthocyanin accumulation increase, we performed two assays: firstly, Chen's assay in order to determine the presence of *lpa1-241* homozygous; secondly, a molecular analysis using *ZmMRP4* sequence-specific amplification polymorphism (S-SAP) markers able to genotype the seeds for the dosage of *lpa1-241* mutation (Figure 3). In all the 125 HIP seeds obtained by selfing *R-sc/R-sc +/lpa1-241* plants, we could verify the tight correlation between the shifted colour and the presence of two doses of *lpa1-241* mutation.

The colour shift is not explained by a change in the anthocyanin species

To test if the colour shift is due to a change of the type of anthocyanin synthesized in the *lpa1-241* seeds, we performed an HPLC analysis on (see Material and methods section). It is well known that maize kernel accumulates mainly cyanidin 3-glucoside (Pascual-Teresa *et al.* 2002) and this was confirmed also by the analysis of our genotypes (Figure 4, peak 1). No remarkable differences were repeatedly noticed in the patterns displayed by *lpa1-241* homozygous (Figure 4A) and wild type control seeds (Figure 4B) exception made for the minor peaks 2 and 3 visible in Figure 4A. However, the size of these peaks, not yet characterized, is not such to explain the strong d ifference observed in embryo colour. HPLC analysis performed on *Lpa1-1* also didn't show any difference in the peaks pattern with respect to the control (data not shown).

lpa1-241 seed treatment with propionate reverts blue phenotype to red phenotype and vanadate treatment of wt seeds resembles *lpa1-241* blue colour

The colour of anthocyanin is known to depend on the pH of the environment in which they are accumulated (de Vlaming *et al.* 1983). Thus, the colour of maize kernel or seedling tissue is red because of the acid pH of the vacuolar lumen where they are accumulated. One possible explanation for the colour shift (from red to blue) of the *lpa1-241* scutellum could be a mislocalization of anthocyanin: cytoplasm (neutral environment) instead of vacuole (acid environment). Since these pigments are synthesized in the cytoplasm, this would imply that they are not transported into vacuole. To test this hypothesis we performed two types of experiments. First of all we used propionic acid to artificially lower the cytosolic pH, secondly we used a vanadate treatment to inhibit the vacuolar pumps. Propionate (20 mM propionate Mes-KOH pH 4) and control (Mes-KOH pH 4) treatments were applied to whole embryos excised from imbibed mature seeds (wt and *lpa1-241* homozygous) and as shown in Figure 5A and 5B the treatments did not affect the red colour of the wt embryo.

On the other hand, propionate treatment of *lpa1-241* embryo caused a marked reversion of the colour from blue to red (Figure 5D), while the control acid treatment did not modify the blue colour (Figure 5C). Conversely, the vanadate treatment did not change the blue colour of *lpa1-241* embryo with respect to the control treatment (Figure 5G, 5H), while the wild type embryo changed the colour from red to blue, mimicking the mutant (Figure. 5E, 5F).

Histological analysis of *lpa1-241* seeds

With the aim to determine the pattern of anthocyanin accumulation in the scutellum, we used histological preparations preserving anthocyanin pigments *in situ*. Histological analyses performed using differential interference contrast imaging microscopy on imbibed mature kernels showed that anthocyanin are accumulated mainly in the glandular layer and secondly in the interior cells of the

scutellum (Figure 6). In the mutant, the cells of the uniformly blue pigmented glandular layer were smaller compared to wild type (Figure 6A, 6B) and in the interior cells of the mutant the blue cells appear smaller compared to the colourless cells (Figure 6C).

In fact, statistical analysis revealed that in *lpa1-241* mutant the cells of glandular layer were smaller by about 75% than the wild type ones (Figure 7A), and the blue cells of the inner layer were about 44 % smaller with respect to the colourless cells (Figure 7B).

Expression analysis of *ZmMRP4* and *ZmMRP3* genes

ZmMRP4 (accession number EF586878) and *ZmMRP3* (AY609318) gene expression levels, responsible, respectively, for the *lpa1* phenotype and the anthocyanin transport, were analyzed in the scutellum and in seedling tissues of wild type and *lpa1-241* mutant by RT-PCR (see Material and methods for details). As shown in Figure 8 and previously reported in our studies (Pilu *et al.* 2009), *ZmMRP4* gene expression was reduced in *lpa1-241* seedlings and was even more reduced in the scutellum compared to the wild type corresponding tissues. The same pattern was observed for *ZmMRP3*. Moreover, *ZmMRP4* gene was more expressed in the scutellum while *ZmMRP3* was more expressed in the seedling.

Discussion

The reduction of phytic acid content in the seeds and the corresponding increase in the level of free phosphorus is a geneticist's goal that in the last twenty years has been approached using traditional and advanced transgenic techniques to tackle the nutritional and environmental problems associated with phytate (reviewed by Raboy 2009). One of the most promising solutions for this problem was achieved by the isolation of recessive mutations causing a big lowering of seed phytic acid content (Larson *et al.* 1998, 2000; Rasmussen and Hatzack 1998; Raboy *et al.* 2000; Wilcox *et al.* 2000; Hitz *et al.* 2002; Pilu *et al.* 2003a; Guttieri *et al.* 2004; Bregitzer and Raboy 2006; Liu *et al.* 2007; Campion *et al.* 2009).

On the other hand, phytic acid has a strong antioxidant activity due to its ability of preventing the formation of several reactive oxygen species (ROS) (Graf *et al.* 1984; Graf and Eaton 1990; Doria *et al.* 2009). In fact, phytic acid may act as an important factor to avoid ageing-related damage to seed embryo and the consequent decrease in germination capacity. Furthermore, for the same reason, it has been shown that, from a nutritional point of view, the presence of phytic acid in the diet of monogastric animals and of man, in particular of the well-nourished populations of developed countries, warrants important health-beneficial effects (Graf *et al.* 1987; Vucenik and Shamsuddin 2006; Raina *et al.* 2008). Therefore, with the aim of compensating for the loss of ROS-scavenging capacity in our maize *lpa1* mutant, by crossing *lpa1-241* and *lpa1-1* we produced near isogenic lines carrying *R-sc* an allele of *R1*. Indeed, several experiments suggest that these molecules act as potent *in vivo* antioxidants offering protection against cancers, cardiovascular diseases and in general chronic degenerative diseases (Renaud and de Lorgeril 1992; Prior 2003; Hou *et al.* 2004; Seeram *et al.* 2004; Toufektsian *et al.* 2008).

In the course of our experimental work on the analysis of anthocyanin content in the *lpa1-1* and most of all in the *lpa1-241* coloured line vs. wt coloured controls, we noticed an increase of pigmentation in the *lpa1* mutant due mainly to a differential accumulation of anthocyanins in the scutellum (Figure 1). Moreover, even in the case of *R-sc/R-sc lpa1-1/lpa1-1,* we observed a slightly (*lpa1-1* mutation is "weaker" compared to *lpa1-241* one) higher anthocyanin content with respect to the control *R-sc/R-sc* +/+ (data not shown).

To our knowledge, this is the first report of an interaction between anthocyanin and phytic acid biosynthetic pathways, the former starting from the amino acid phenylalanine and the latter from the cyclic polyalcohol *myo*-inositol. The increase of anthocyanin content can be observed mainly in the scutellum (Figure 1), a tissue where phytic acid is accumulated at high level (O'Dell *et al.* 1972). To explain this increase in seed anthocyanin content, it may be conjectured that the negative pleiotropic effects observed in the *lpa1-241* mutant (Pilu *et al.* 2005; Doria *et al.* 2009) may cause an induction of anthocyanin biosynthesis too. In fact, in

many cases, the accumulation of anthocyanin in plant tissues may be a marker of several plant stresses such as oxidative damage, harmful radiation, pathogen infection etc. (reviewed by Winkel-Shirley 2002). Apart from these quantitative aspects regarding anthocyanins, we also observed that the scutellum colour of *lpa1-241* mutant appears bluish (Figure 2A) and not red as the control (Figure 2B), whilst the seedling of the same mutant maintain the red coloration displayed by the control (Figure 2C). We observed a strong difference in seedlings size between wt and mutant due to pleiotropic effects exhibited by *lpa1-241* mutation as described in our previous works (Pilu *et al.* 2005; Pilu *et al.* 2009). The correlation between the *lpa1-241* mutation and the anthocyanin colour shift was confirmed in all the embryos analysed using a molecular marker (Figure 3). Furthermore, HPLC analysis suggested that the bluish colour observed in the scutellum is not due to a new anthocyanin compound. We observed indeed the occurrence of unidentified compounds in the mutant (Figure 4, peaks 2 and 3), but they would be in amounts too tiny to account for the above described phenomenon.

In several plants, the coloration of tissues depends on the accumulation of pigments in the vacuoles of cells and their absorption spectrum depends on the pH of the vacuolar environment (de Vlaming *et al.* 1983; Yoshida *et al.* 1995). Taking into account that a basification of the vacuole sufficient to drive a shift colour from red to blue would probably be too severe to be compatible with cell survival, we conjectured that in our system a mislocalization of anthocyanins in the cytoplasm due to a reduction of vacuolar anthocyanin transport is much more probable. In fact as shown in Figure 5, while propionate treatment did not cause any effect on wt (Figure 5A, 5B), an inversion of colour (from blue to normal red colour) was observed in the *lpa1-241* mutant embryos. These data strongly suggest the permanence of anthocyanins in the basic-neutral cytoplasm environment, which causes the colour shift to blue. Vanadate treatment did not exhibit any effect on *lpa1* blue embryo (Figure 5G, 5H) while treatment of wt embryo causes a shift colour from red to blue, mimicking the mutation colour (Figure 5E, 5F) and further supporting the working hypothesis. On the whole, these data suggest that

lpa1-241 mutant has a decreased capacity of transporting anthocyanins into the scutellum, but not into seedling vacuoles, as shown in Figure 2C.

Histological analyses carried out while preserving the natural tissue pigmentation showed that anthocyanins are mainly localized in the globular layer of the scutellum (Figure 6A, 6B) and, in a disordered way, in the inner layer (Figure 6C). Furthermore, the mutant cells dimensions in the globular layer appear smaller compared to wt and in the inner layer of the mutant blue cells were smaller compared to the colourless ones (Figure 6C). The strong effect of the *lpa1-241* mutation on cells size is not surprising, in fact InsP metabolism plays important roles in several crucial process such as signal transduction (Stevenson *et al.* 2000).

These data indicate that the anthocyanin colour shift from red to blue is an useful and reliable marker to monitor the strength of the *lpa1* mutation and consequently the *ZmMRP4* gene expression at the cellular level. In point of fact, *lpa1-241* mutation was caused by a silencing of *ZmMRP4* gene (Pilu *et al.* 2009) and the correlation between reduced cell dimension and blue cell colour (Figure 7) confirms the strong negative pleiotropic effect caused by the *lpa1-241* mutation on germination and whole plant viability (Pilu *et al.* 2005).

The stochastic distribution of coloured cells in the inner layer (Figure 6C) could reflect the epigenetic origin of *lpa1-241* mutation as previously reported for several regulatory genes displaying somatic variability with high frequency (reviewed in Chandler *et al.* 2000).

It is known that anthocyanins are synthesized in the cytoplasm and transported into the vacuole by involvement of *ZmMRP3* protein (Goodman *et al.* 2004). In order to verify a possible correlation between *ZmMRP3* expression and the colour shift observed in the *lpa1-241* embryo we performed an RT-PCR analysis (Materials and methods for details). As shown in Figure 8, *ZmMRP3* mRNA level was lower in the *lpa1-241* mutant tissues of both embryo and seedling, while in the case of *ZmMRP4* mRNA levels we observed the same pattern previously registered in *lpa1-241* seedlings (Pilu *et al.* 2009). The reduction in *ZmMRP3* expression level could be explained by an aspecific cell suffering as inferred by the

smaller dimensions of the blue cells. Otherwise, it might be caused by a coregulation of *ZmMRP4* and *ZmMRP3* genes.

In summary, to explain the colour shift observed in the scutellum, three hypotheses may be proposed:

(i) the decrease in the *ZmMRP3* expression level, one of the putative anthocyanin transporters, causing a cytoplasmic mislocalization of anthocyanin.

In fact, Goodman *et al.* (2004) using antisense technology found that *ZmMRP3* gene silencing caused a mislocalization of anthocyanin content driving a colour shift from red to burnished in plant tissues, while no change was observed in the kernel aleurone layer. In our system, we observed a change from red to bluish due to the presence of *R-sc* allele in the scutellum, a tissue not analysed in the Goodman's paper (2004). This first hypothesis is supported also by the mutant expression analysis (Figure 8) showing a much higher level of *ZmMRP3* expression in seedling compared to embryo, thus explaining the absence of a colour shift in the seedling tissue (Figure 2C);

(ii) *ZmMRP4* acts also as an anthocyanin transporter. We may suppose that in the scutellum the *ZmMRP4* transporter is the main anthocyanin transporter and in seedling and mature plant this activity is carried out by *ZmMRP3.* In point of fact, our expression analysis showed that *ZmMRP4* is more expressed in the embryo whilst *ZmMRP3* in the seedling tissues;

(iii) *ZmMRP4* and *ZmMRP3* act both as InsP (*myo*-inositol phosphates) and anthocyanin transporters. Although the function of *ZmMRP3* and *ZmMRP4* genes could be partially redundant, *ZmMRP4* could act mainly as InsP transporter in the seed whilst *ZmMRP3* could act mainly as anthocyanin transporter in the green tissues.

Supporting this idea is the fact that several genetic screenings have failed to isolate an aleurone anthocyanin transporter suggesting an essential role for *ZmMRP3* and/or the presence of another transporter. Furthermore, Goodman *et al.* (2004) using a strong CaMV35S promoter, failed to recover antisense plants indicating a possible lethal effect caused by the complete absence of ZmMRP3 protein.

In conclusion, this is the first report of an interaction between anthocyanin and phytic acid accumulation in plant seeds, leading to an increase of anthocyanin content and a colour shift in the scutellum coloration. This interaction could help to discover the carriers and the regulation mechanisms involved in the vacuolar transport of plant cell and xenobiotic molecules involved in several fundamental cell processes, not so far fully understood.

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Figures

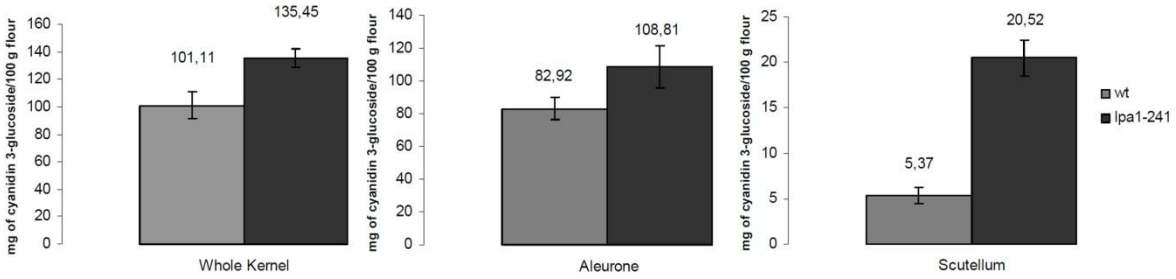

Figure 1. Whole kernel, aleurone and scutellum anthocyanin content in *R-sc/R-sc* +/+ control and *R-sc/R-sc lpa1-241/lpa1-241* genotype. Mean values represent at least ten independent replicates. Confidence intervals at 95% are shown.

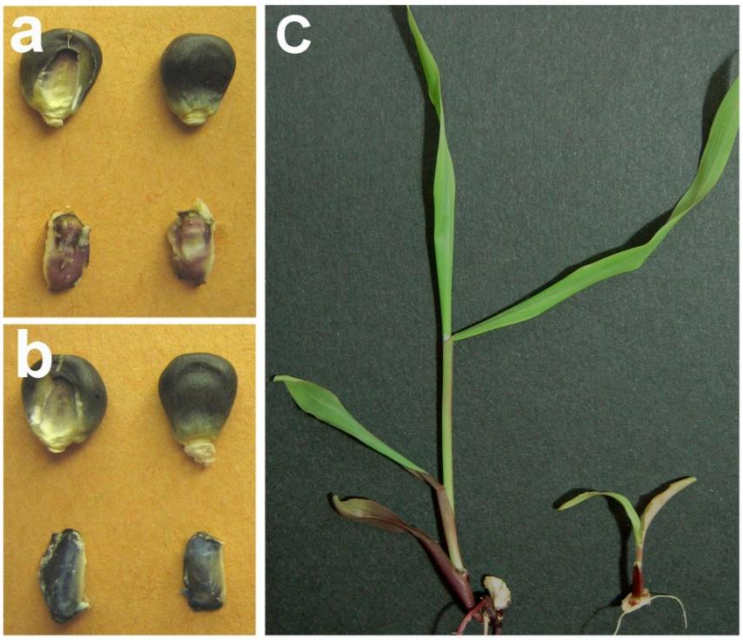

Figure 2. Anthocyanin pigmentation of whole kernel (above) and scutellum (below) in *R-sc/R-sc* +/+ control genotype (**a**) and *R-sc/R-sc lpa1-241/lpa1-241* genotype (**b**). (**c**) Anthocyanin pigmentation of *Sn:bol3/Sn:bol3* +/+ seedling (left) and *Sn:bol3/Sn:bol3 lpa1-241/lpa1-241* seedling (right). Whole embryos were excised from mature seeds imbibed in sterile deionized water for 18 h and the seedlings were obtained after 9-10 days of growth on MS medium

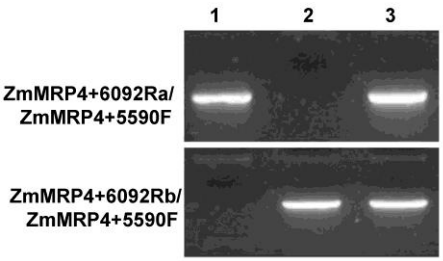

Figure 3. *Lpa1* allele molecular genotyping. *lpa1-241/ lpa1-241* (lane 1), +/+ B73 (lane 2) and *+/lpa1-241* (lane 3) allele specific amplified products are shown obtained using respectively ZmMRP4+6092Ra (*lpa1-241* allele) and ZmMRP4+6092Rb (+ B73 allele) specific forward primers.

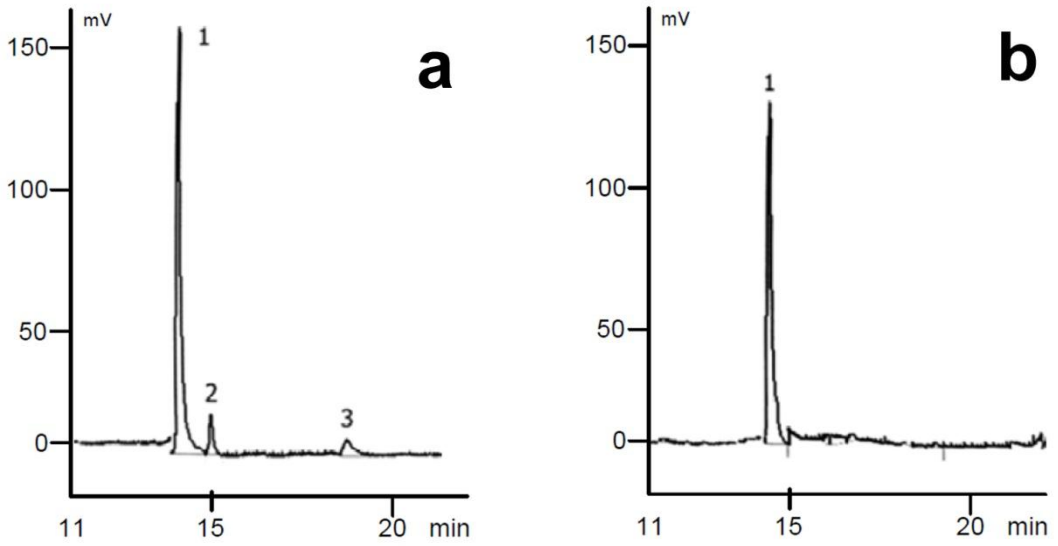

Figure 4. HPLC profile of anthocyanin extracted from *R-sc/R-sc lpa1-241/lpa1-241* (**a**) and *R-sc/R-sc* +/+ control seeds (**b**). The peak number 1 corresponds to cyanidin 3-glucoside, the peaks number 2 and 3 are still uncharacterized.

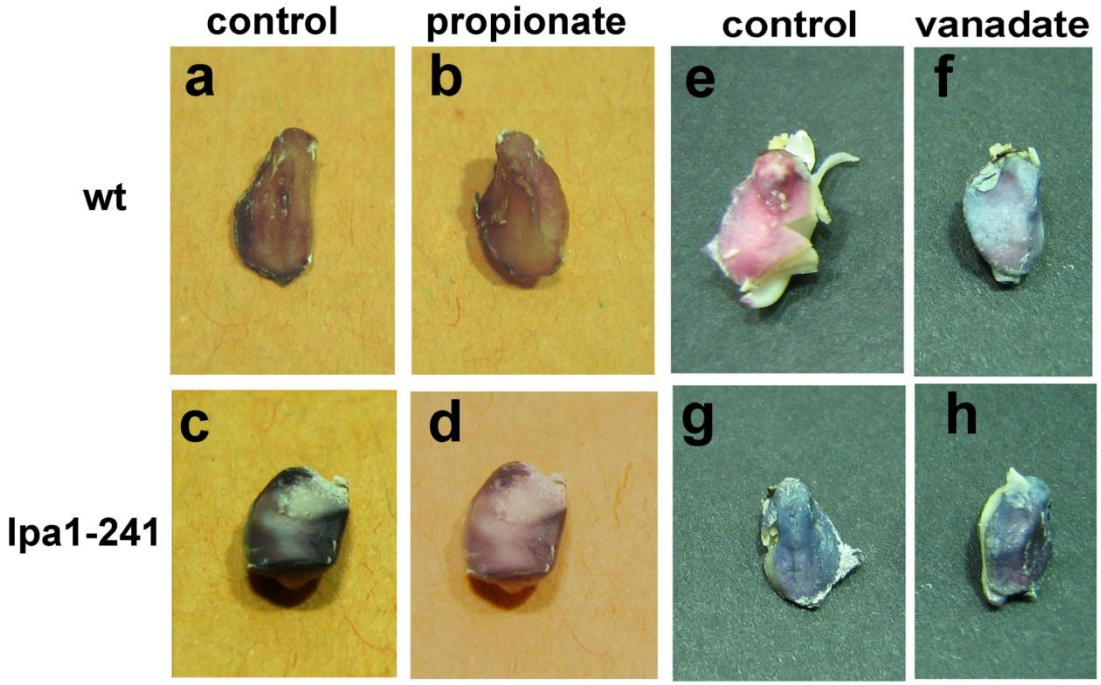

Figure 5. Embryo propionate and vanadate treatments of wild type (*R-sc/R-sc* +/+ genotype) and *lpa1-241* (*R-sc/R-sc lpa1-241/lpa1-241* genotype) mutant. Treatment of wild type with pH 4 control buffer (**a**) and pH 4 propionate buffer (**b**). *lpa1-241* treatment with pH 4 buffer control (**c**) and pH 4 propionate buffer (**d**). Images were taken after 2 hours of incubation. Treatment of wild type with control buffer (**e**) and with vanadate buffer (**f**). *lpa1-241* treatment with control buffer (**g**) and vanadate buffer (**h**). Images were taken after 48 hours of incubation.

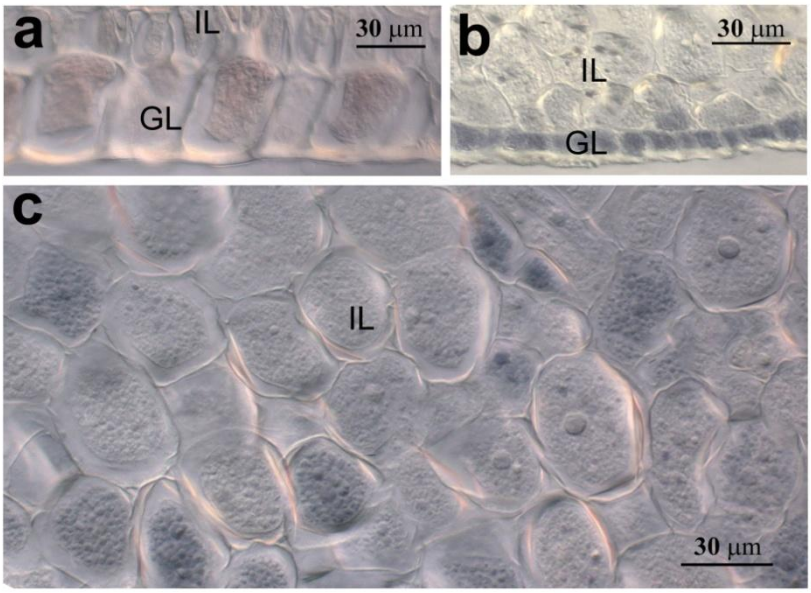

Figure 6. Histological analyses of scutellum preserving anthocyanin pigments *in situ*. (**a**) Glandular layer of wild type (*R-sc/R-sc* +/+ genotype) and (**b**) mutant (*R-sc/R-sc lpa1-241/lpa1-241*). (**c**) mutant inner layer. GL, glandular layer; IL, inner layer.

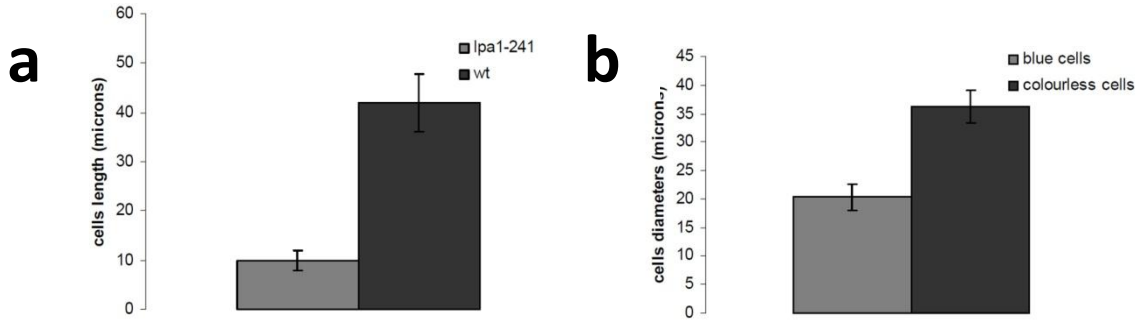

Figure 7. Cell dimensions (major axis) of scutellum glandular layer in wild type and *lpa1-241* mutant (**a**). Cell diameters of scutellum inner layer of blue and colourless cells in *lpa1-241* mutant (**b**). Confidence intervals at 95% are shown.

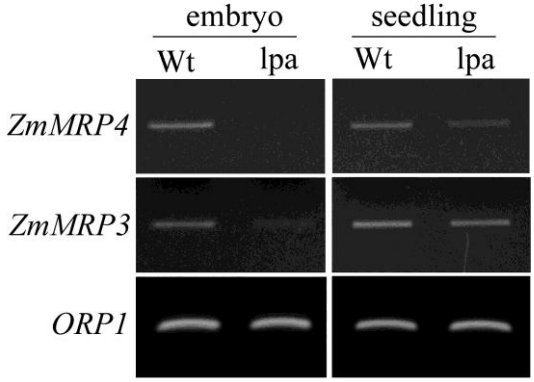

Figure 8. RT-PCR analysis showing the expression of *ZmMRP4* and *ZmMRP3* genes in embryo and seedling of wild type (*R-sc/R-sc* +/+ genotype) and *lpa* (*R-sc/R-sc lpa1-241/lpa1-241* genotype) mutant. *Orp1* gene amplification is shown as control.

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