Heredity (2009) 102, 236–245

www.nature.com/hdy

ORIGINAL ARTICLE

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

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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 (Ins)-3-phosphate synthase gene (*mips1S*). In this study, we present 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 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.
Heredity (2009) **102,** 236–245; doi:10.1038/hdy.2008.96; published online 10 September 2008

Keywords: maize; phytic acid; 5-Azacytidine treatment; gene silencing; paramutation

Introduction

Phytic acid (*myo*-inositol (Ins)-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, 1990). The biosynthetic route begins with Ins and can be summarized as showed in Figure 1. *Myo*-Ins, 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*-Ins 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*) and involved in this pathway (Figure 1). They produce seeds with reduced phytic acid content and a proportionally higher level of free phosphate, whereas 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 EF86878), coding a multidrug resistance-associated-protein (MRP) mapping near the *mips1s* sequence, is the actual responsible gene for *lpa1* mutation (Shi *et al*, 2007). MRP proteins represent a subfamily of ATP-binding cassette transmembrane transporters widespread in all eukaryotes, which in plants are involved in several functions, such as xenobiotic detoxification, organic ions transport, oxidative stress tolerance (Swarbreck *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*

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Received 26 March 2008; revised 1 July 2008; accepted 8 August 2008; published online 10 September 2008

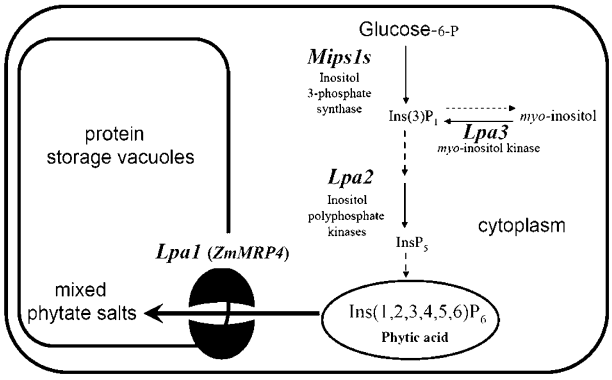

Figure 1 Schematic model of the biosynthetic pathways leading to phytic acid accumulation in protein storage vacuoles of the maize seed. Main known genes involved in inositol (Ins) phosphate pathway are shown. *Mips1S* encodes for a *Myo*–Ins(3)P1 synthase, which converts glucose-6-P to Ins(3)P1. Ins(3)P1 can be produced also by the action of a *myo*-Ins kinase encoded by *Lpa3* gene. *Lpa2* encodes an Ins phosphate kinase, which along with other kinases leads to phytic acid, Ins(1,2,3,4,5,6)P6 synthesis. *Lpa1* gene (*ZmMRP4*) is a transmembrane transporter hypothesised to load phytate into protein storage vacuoles.

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 it 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, whereas *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 study, 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 (for example, *Pl′*, *B′* and so on). In this study, 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 (inbred line homozygous dominant for the colour factors *A1*, *C1* and *R1*) are named $Lpa1_{ACR}$, whereas 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) 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 1 h 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 h. After 1 h at room temperature, the free phosphate content was quantified through the use of a spectrophotometer ($\lambda = 650$ 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 1 h at room temperature, and then 800 µl of Chen's reagent was added. After 1 h 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′-AATC AAGACGATGAGAAAAGTTAT-3′), whereas B73 specific primer was ZmMRP4 + 6092Rb (5′-AATCAAGACG ATGAGAAAAGTTC-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 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 63 °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 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 were 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 °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 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 h (in order to obtain mature plants), or at 30 °C for 18 h (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, St Louis, MO, USA, 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 6–14 days and shoot tissue was sampled and stored at –80 °C 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, USA). 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.

Reverse transcriptase-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). 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 U μg^{-1}) 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 1.25 U 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 °C for 1 min, extension at 72 ° for 1.5 min. Extension at 72 °C 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′-AAGGACGTGCA CACCGC-3′ and downstream primer, 5′-CAGATACAGA ACAACAACTC-3′. The length of the amplified product was 207 bp. Several cycles of successive cDNA dilutions and *orp1* amplification were carried out 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′-GTTCCCTTCCAGCAG CTAAC-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′-TCATGG TGTAAGTTGTATGTTTC-3′) and ZmMRP4 + 6206R (downstream primer 5′-CTTCTCTATATACAGCTCGAC-3′). A 677 bp amplificate is obtained 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. Final extension at 72 °C 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 ultraviolet light.

Results

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

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 milligram of atomic P per gram 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 allele 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*., 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 of this behaviour, we crossed heterozygous ACR *Lpa1*/*lpa1-241* families with wild-type plants from the B73 inbred line and observed that

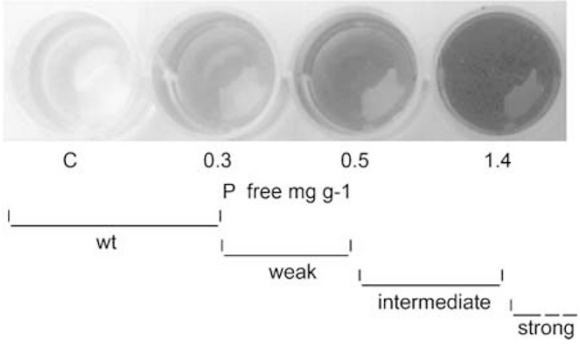

Figure 2 Assay to detect *lpa1-241* phenotypic classes. Single seeds were crushed, extracted and assayed for free P using a microtitre plate-based colorimetric assay (Chen *et al*., 1956). Classes detectable by visual inspection were: wild type (wt) 0–0.3; weak (W) 0.3–0.5; intermediate (I) 0.5–1.4;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

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 F_3 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 $lpa1\text{-}241_{ACR}$ 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 $Lpa1_{B73}$/$lpa1\text{-}241_{ACR}$, $Lpa1'_{B73}$/$lpa1\text{-}241_{ACR}$ and $Lpa1''_{B73}$/$lpa1\text{-}241_{ACR}$ 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 gram of flour in average, whereas subsequent progeny from these homozygous *Lpa1′* plants produced seeds with lower free phosphate levels: 0.20 mg g^{-1} for F_3 and 0.14 mg g^{-1} 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

To directly detect $Lpa1_{B73}$ allele silencing, heterozygous $Lpa1_{B73}$/*lpa1-241* 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).

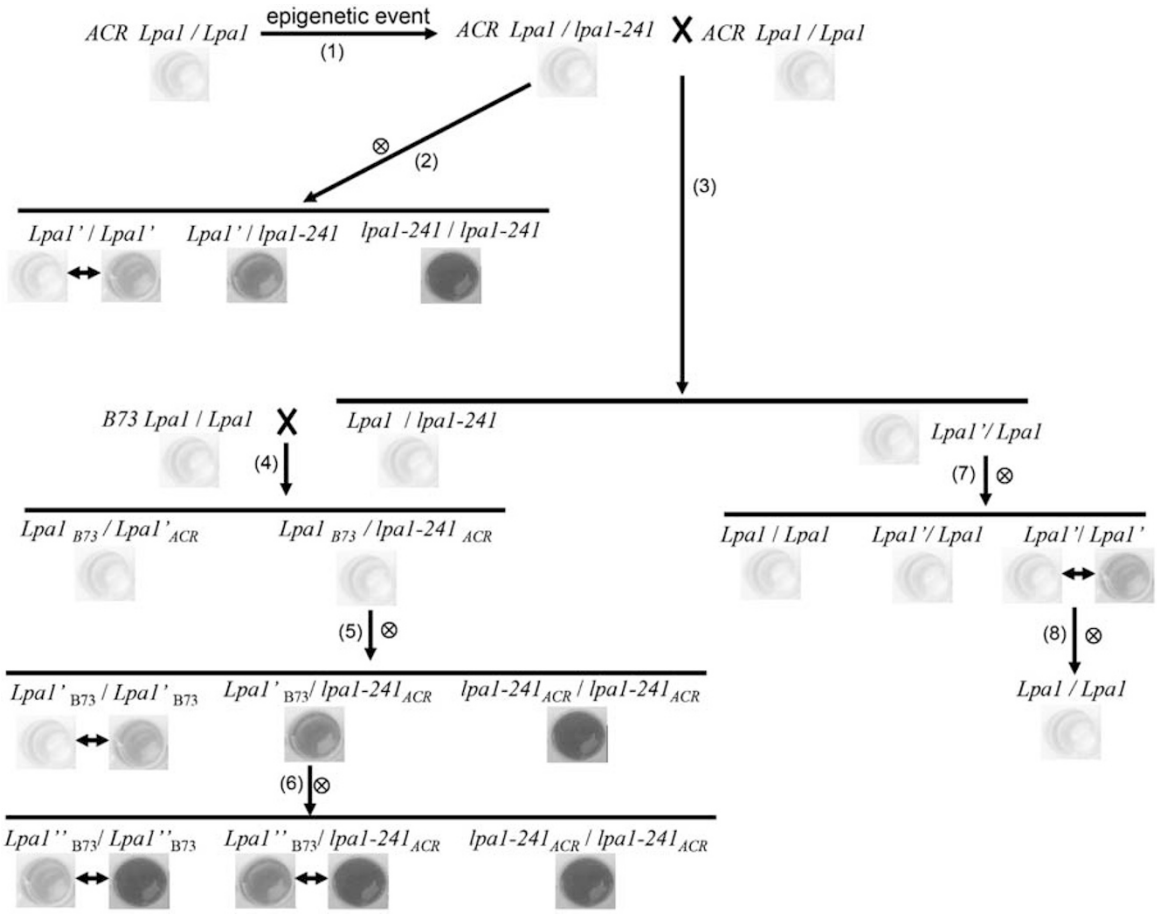

Figure 3 Diagram of *lpa1-241* mutation pedigree. A *Lpa1* wild-type allele spontaneously underwent silencing in ACR inbred line carrying the colour factors *A1*, *C1* and *R1* (1). The event was named *lpa1-241* and its phenotype was detected in the progeny obtained by selfing *Lpa1/lpa1-241* ACR plant (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 and 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 with genotype is also shown. Genotypes have been determined by specific *ZmMRP4* allele molecular marker.

The same experiment was conducted with plants from W64A, K6, H99 and W22 inbred lines. An F_1 family of each genotype, $Lpa1_{W64A}$/*lpa1-241*, $Lpa1_{K6}$/*lpa1-241*, $Lpa1_{H99}$/*lpa1-241* and $Lpa1_{W22}$/*lpa1-241* F_1 was crossed with a B73 *lpa1-1/lpa1-1* family. Each cross progeny was analysed 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

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 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 F_1 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

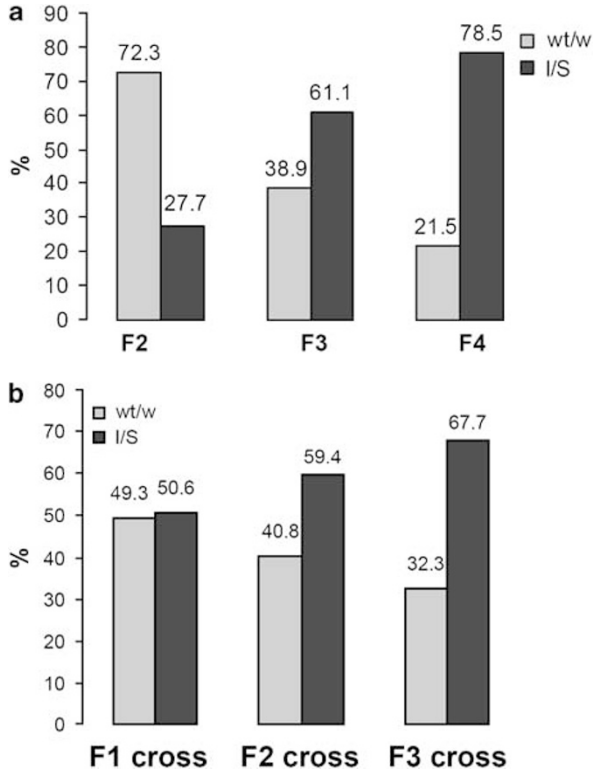

Figure 4 Changes in phenotypic class segregation ratios over generations in the presence of *lpa1-241* allele. (**a**) Seed free phosphate phenotypic classes are presented in histogram as a percentage of occurrence in selfed heterozygous progenies, where F_2 refers to *Lpa1/lpa1-241* selfed progeny, 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* × *lpa1-1/lpa1-1* progeny, F_2 cross refers to *Lpa1′/lpa1-241* × *lpa1-1/lpa1-1* progeny, F_3 cross refers to *Lpa1″/lpa1-241* × *lpa1-1/lpa1-1* progeny. The sum of wild-type and weak (wt/w) phenotype occurrence (grey bars) vs intermediate plus strong (I/S) phenotype occurrence (black bars) are shown. Percentage values are shown at the top of each bar.

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 seed 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 with 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 Murashige and Skoog (MS) medium. These seeds treated with 5-Azacytidine always showed a remarkable increase in growth (of about 50%) compared with 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 analysed in seedling tissues by RT-PCR.

Table 1 Effects of generation on heritability of high inorganic phosphate phenotype (HIP) phenotype in *lpa1-241* lines

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%)

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).

Table 2 Effects of generation on heritability of high inorganic phosphate phenotype (HIP) phenotype in *lpa1-241* lines

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%)

$Lpa1_{B73}$/*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).

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 with 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 with untreated mutants of the same phenotypic class (Figure 8c, lane 3 *vs* lane 4), whereas 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 with 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).

Reverse transcriptase-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).

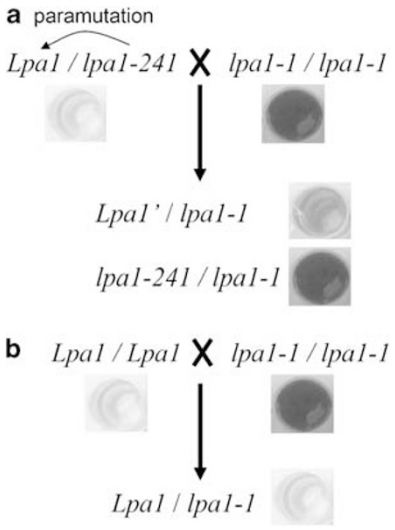

Figure 5 *Lpa1* allele silencing test after exposure to *lpa1-241* paramutagenic allele. Heterozygous *Lpa1/lpa1-241* families have been used in crosses with homozygous *lpa1-1* mutant as a test for *Lpa1* alleles activity. (**a**) Phenotypes obtained crossing *lpa1-1/lpa1-1* with a *Lpa1/lpa1-241* F_1 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*.

Discussion

The *lpa1-241* recessive mutation was isolated in an ethyl methane sulphonate 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 $lpa1\text{-}241/Lpa1_{B73}$ heterozygotes (Figure 3, crosses 5 and 6).

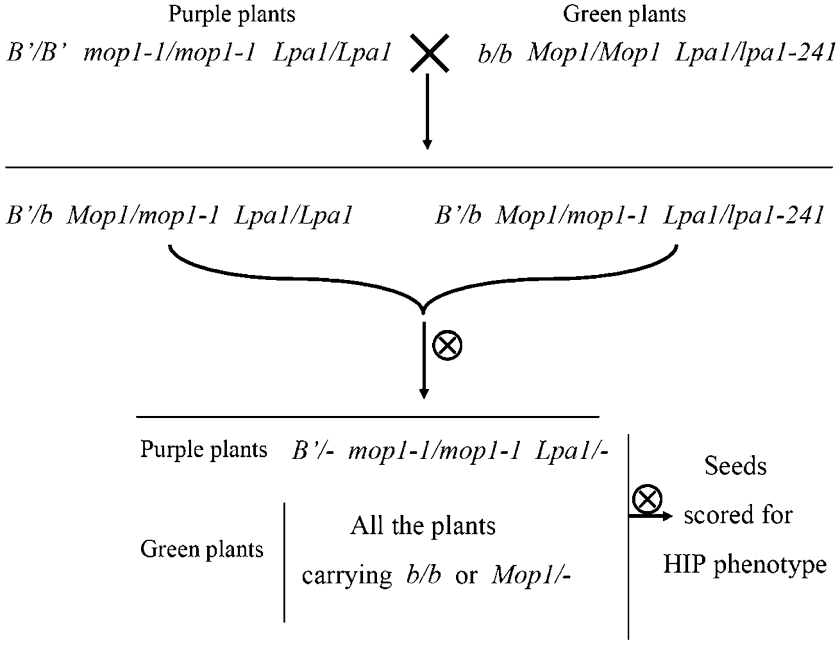

Figure 6 Diagram showing the crosses between *mop1-1* and *lpa1-241* mutants and the genotypes tested. Homozygous *mop1-1* purple plants (*B'/B' mop1-1/mop1-1 Lpa1/Lpa1*) were crossed with heterozygous *lpa1-241* green plants (*b/b Mop1/Mop1 Lpa1/lpa1-241*), the progeny was selfed and seeds obtained 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.

As observed in ACR families, in F_2, F_3 and F_4 generations where *lpa1-241* allele was present, seed free phosphate levels increased, suggesting a reduction in the 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″*, and so on) 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).

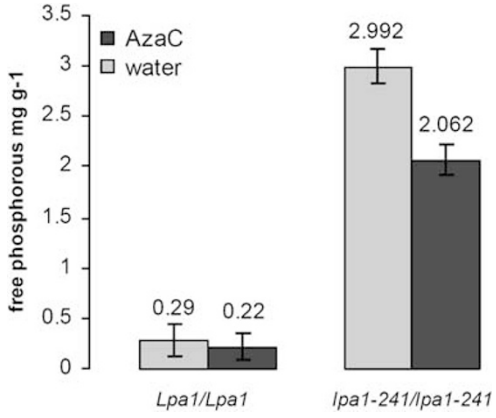

Figure 7 Seed free phosphorus content of 5-Azacytidine-treated *lpa1-241* seeds progeny. Average seed free phosphorus content (milligram P per gram of flour) of *Lpa1/Lpa1* B73 and homozygous *lpa1-241/lpa1-241* individuals from *lpa1-241* segregating families. Black bars refer to 5-Azacytidine-treated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

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

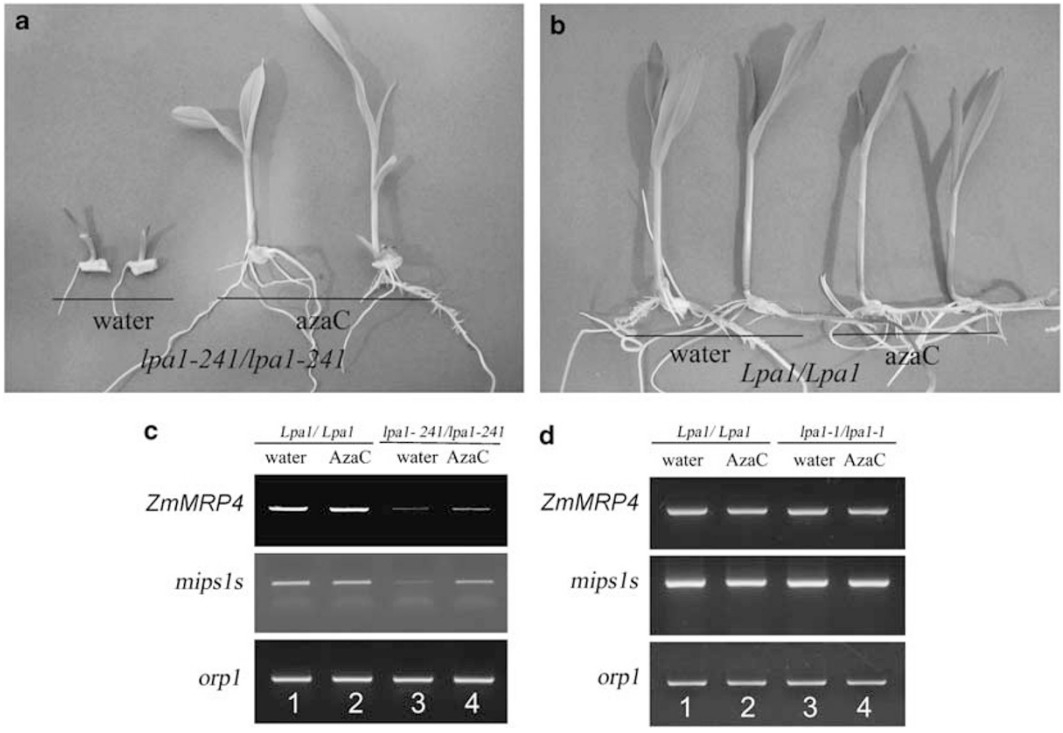

Figure 8 Effects of seeds 5-Azacytidine treatment on *lpa1-241* seedlings growth *in vitro* and on *mips1s* and *ZmMRP4* genes expression. (**a**) Fourteen days old homozygous *lpa1-241* seedlings, untreated (water) and treated (AzaC). (**b**) Six days old *Lpa1/Lpa1* B73 seedlings, untreated (water) 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**) Reverse transcriptase-PCR (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 with homozygous *lpa1-1* (untreated, lane 3; treated, lane 4). *Orp1* gene amplification is also shown.

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), whereas 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 with 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), whereas 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 with 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 with wild type, whereas 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*-Ins 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 because of 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 do not know whether 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 can significantly revert *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

This study was supported by Fondo Interno Ricerca Scientifica e Tecnologica (F.I.R.S.T. 2004, 2005 and 2006 to R Pilu). We thank Dr Victor Raboy, USDA ARS, Aberdeen, ID, USA, for his generous gift of *lpa1-1* seeds, the anonymous referees for their precious comments and advices and Dr Davide Reginelli for his hard work in the field.

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