Theor Appl Genet (2003) 107:980–987
DOI 10.1007/s00122-003-1316-y

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Phenotypic, genetic and molecular characterization of a maize low phytic acid mutant (*lpa241*)

Received: 25 October 2002 / Accepted: 26 March 2003 / Published online: 14 June 2003

Abstract Phytic acid, *myo*-inositol 1,2,3,4,5,6-hexakisphosphate, is the major storage compound of phosphorous (P) in plants, predominantly accumulating in seeds (up to 4–5% of dry weight) and pollen. In cereals, phytic acid is deposited in embryo and aleurone grain tissues as a mixed "phytate" salt of potassium and magnesium, although phytates contain other mineral cations such as iron and zinc. During germination, phytates are broken down by the action of phytases, releasing their P, minerals and *myo*-inositol which become available to the growing seedling. Phytic acid represents an anti-nutritional factor for animals, and isolation of maize *low phytic acid* (*lpa*) mutants provides a novel approach to study its biochemical pathway and to tackle the nutritional problems associated with it. Following chemical mutagenesis of pollen, we have isolated a viable recessive mutant named *lpa 241* showing about 90% reduction of phytic acid and about a tenfold increase in seed-free phosphate content. Although germination rate was decreased by about 30% compared to wild-type, developement of mutant plants was apparentely unaffected. The results of the genetic, biochemical and molecular characterization experiments carried out by SSR mapping, MDD-HPLC and RT-PCR are consistent with a mutation affecting the *MIPS1S* gene, coding for the first enzyme of the phytic acid biosynthetic pathway.

Communicated by F. Salamini

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Keywords Maize · Mutagenesis · Phytic acid · *Myo*-inositol(3) P1 synthase · RACE

Introduction

Plant seeds are the major source of nutrients for humans and animals. With regard to phosphorous (P), however, although seeds contain a wealth of this element, in nearly all plant species up to 80% of it is bound to phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phospate, or Ins P6), a molecule virtually indigestible by, and thus not bioavailable to, monogastric animals (Raboy et al. 1990, 1997).

Phytic acid typically represents 4–5% of the seed weight, constituting the reserve of both phosphate and various important mineral cations such as calcium, magnesium, potassium, iron and zinc, which bind phosphate groups to form phytate salts, accumulating as inclusions called "globoids" in seed-protein bodies. It has been estimated that about 50% of the total elemental P applied every year in world agriculture ends up in the phytic acid molecule (Lott et al. 2000).

In cereal grains, essentially all phytate is localized in the embryo and aleurone tissues (O'Dell et al. 1972). Germinating seeds are able to re-extract phosphate as well as cations from phytate thanks to the activity of phytase, an enzyme found also in many microorganisms, but not in non-ruminant animals (Labourè et al. 1993).

Although inositol phosphates are important, ubiquitous and multi-functional molecules, the biosynthetic pathway leading to phytic acid has not yet been thoroughly defined, especially with respect to the sequential phosphorylation steps and the regulative mechanisms. The biosynthetic route can be summarized as consisting of two parts: the early "Ins-supply" pathway and the later Ins phosphate/Ptd Ins-phosphate pathway that converts Ins to InsP6 (Fig. 1). The only known synthetic source for the Ins ring is the activity of the enzyme D-*myo*-Inositol(3)P1 synthase (MIPS), that converts glucose 6-P to D-Ins(3)P1; this molecule may be directly phosphorylated to yield Ins P6, or may first be hydrolysed via a specific monophos-

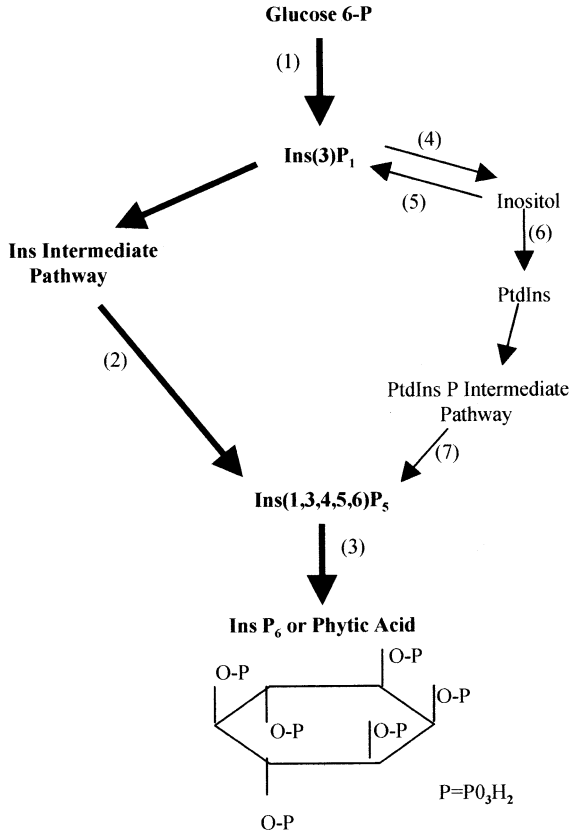

Fig. 1 Plant biosynthetic pathways leading to phytic acid can be summarized as consisting of two parts: Ins supply and subsequent Ins Polyphosphates synthesis. (1) Ins(3)-P1 synthase (MIPS); (2) InsP- Kinases or PolyP kinases; (3) Ins(1,3,4,5,6) P5 2-Kinase; (4) Ins 3-phosphatase; (5) Ins 3-Kinase; (6) PtdIns synthase; (7) PtdIns and PtdIns P kinases

phatase to yield free Ins and inorganic P. As well as being phosphorylated to phytic acid, the *myo*-inositol moiety is the precursor for many other compounds playing a central role in several metabolic processes (Loewus and Murthy 2000).

There seems to be a close relationship between the biosynthesis of phytic acid and the formation of D-*myo*-inositol-3-phosphate by MIPS. Northern-blot analysis and in situ hybridization of a rice cDNA (*RINO1*) highly homologous to the MIPS of yeast and several plant species showed that high levels of transcripts accumulated in the embryo, but were undetectable in shoot, roots and flowers (Yoshida et al. 1999). Also, in soybean, transcripts encoding MIPS (*Gm*MIPS1) were mainly located in developing seeds, only during the early phase of seed development (Raboy and Dickinson 1987).

The maize genome has up to seven loci containing sequences with high homology to yeast and other plant MIPS genes (Larson and Raboy 1999). However, no studies regarding the tissue specificity of these different maize MIPS sequences has been carried out so far.

In order to throw light upon phytate genetics and metabolism, a convenient approach consists in selecting low-phytic-acid mutants (*lpa*) defective in phytic-acid biosynthesis and, therefore, accumulating high levels of free phosphate in their seeds. Such mutants, producing seeds in which the chemistry of seed P, but not the total amount of P, is greatly altered, have indeed been isolated in maize (up to 65% reduction in grain phytic-acid content), barley (up to 75% reduction), rice (up to 45% reduction) and soybean (Raboy and Gerbasi 1996; Larson et al. 1998, 2000; Rasmussen and Hatzack 1998; Raboy et al. 2000; Sebastian et al. 2000).

In cereals, the *lpa* mutations so far selected belong to two categories: *lpa1*, leading to the exclusive increase of free phosphate, and *lpa2*, accumulating free phosphate, but also inositol phosphates with up to five PO_4 residues. Regarding maize *lpa1* mutants, there is evidence indicating they all map on chromosome 1S, where a *MIPS* locus has also been localized (Raboy et al. 2000). Concerning the applicative aspect, although a few breeding programs are using the already available *lpa* mutants to develop low phytate crops, additional biochemical as well as agronomic and breeding studies are required with newer mutant materials (Raboy et al. 2001).

In the present work we report: (1) data concerning the isolation and characterization under different aspects of a maize *lpa* mutant still viable in the homozygous state in spite of a 90% reduction in grain phytic acid level, and (2) the first molecular evidence for the involvement of the *MIPS1S* gene in *lpa1* phenotypes.

Materials and methods

Mutant isolation and genetic analysis

The ACR seed stock used in this study was homozygous dominant for the colour factors *R, a1, a2, c1, c2, bz1* and *bz2*, and homozygous recessive for the *b1* gene.

Starting from the above stock, a population of EMS (ethyl-methane-sulfonate)-induced mutants was generated using the pollen-treatment method (Neuffer and Coe 1978). Treated pollen produced M1 seeds heterozygous for the induced mutation, these were planted and self-pollinated to produce 600 M2 ears. The first screening for the HIP phenotype was carried out by the molybdate staining assay (see Quantitative Determination of the Seed P and Inositol P fraction).

Assay for high-phosphate (HIP) levels in the kernel of maize

An indirect but simple-screening procedure for detecting putative low-phytate maize mutants was adopted from a study on maize low-phytate mutants by Raboy et al. (2000). The rationale behind this screening is that a high free phosphate level is indicative of a mutant affected in the phytic acid synthesis since phosphate is likely to accumulate when phytic acid is not, or is, produced in a reduced amount. About 600 M2 families were screened for phosphate content.

Twenty seeds were sampled from each M2 ear and individually ground in a mortar with a steel pestle. One hundred milligrams of the resulting flours were then extracted with 1 ml of 0.4 M HCl at 4 °C overnight. Samples were mixed briefly and 100 μl were removed and supplemented with 900 μl of Chen's reagent [6N 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 the case of high phosphate content, a dark-blue coloured phospho-

molybdate complex formed in 1–2 h. Putative *lpa* mutant grains were then scored for phytate and ortophosphate levels using TLC analysis carried out as described by Rasmussen and Hatzack (1998).

Quantitative determination of the Seed P and Inositol P fraction

Seed total P was determined following wet-ashing of flour aliquots (50–150 mg) and colorimetric assay of digested P (Chen et al. 1956).

A modification of the ferric precipitation method was used for quantitative determination of the Inositol P fraction (Raboy 1990). Samples of dried mature seeds were ground and stored in a dessicator until analysis. Samples of flour (50–200 mg) were extracted in 3 ml of 0.4 N HCl containing 0.7 M Na_2SO_4 with magnetic stirring (room temperature, overnight). Following centrifugation (13,000 rpm for 10 min), an aliquot of supernatant was placed in a Corex tube and a solution of 15 mM $FeCl_3$:0.2 N HCl was added. The mixture was placed in a boiling water bath for 30 min. The ferric phytate precipitate obtained after centrifugation (13,000 rpm for 10 min) was washed with 0.2 N HCl, digested to completion on a hot plate with H_2SO_4 and H_2O_2 as needed, and diluted with distilled H_2O. Phytic acid phosphorous in the digests was determined colorimetrically (Chen et al. 1956).

lpa241 mapping

In order to position *lpa241*, crosses of heterozygous *Lpa24l*/*lpa241l* females to a stock carrying the TB-A translocation (Beckett 1978) were used. The F_1 revealing the HIP phenotype was obtained from crosses involving the TB-1S male parent, thus indicating that *lpa241* lies on the short arm of chromosome 1S. *Lpa241* was then mapped in segregating F_2 populations using SSRs. F_2 seeds were obtained from the selfing (ACR *Lpa241*/*lpa241* × B73) plants. The seeds were cut in two halves and one half was crushed and assayed for the HIP phenotype to establish the genetic constitution, either *lpa241*/*lpa241, Lpa241 lpa* 241 or *Lpa241*/*Lpa241*, of the F_2 seeds, while the other half was used for DNA extraction (Dellaporta et al. 1983). Polymerase chain reactions (PCRs) were performed using SSR primers defined in the Maize Database corresponding to chromosome 1S. Reaction and gel running conditions were as described in the SSR Methods Manual by the Missouri Maize Project (http://www.agron.missouri.edu/ssr.html). Recombinant values were converted to map distances using MAPMAKER 3 (Lander et al. 1987).

Trichloroacetic acid (TCA) extraction of Inositol phosphates and MDD-HPLC analysis

Extraction was performed by suspending 40-mg portions of flour prepared as above described in a 10-fold excess of ice-cold TCA extraction buffer [10% (W/v) TCA/5 mM and NaF/5 mM EDTA]. Samples were agitated for 1 h at 4 °C and centrifuged at 5,000 g for 5 min at 4 °C. Pellets were re-suspended again in TCA extraction buffer and agitated by magnetic stirring at room temperature for another 1.5 h. Supernatants from both extraction rounds were pooled and TCA was largely removed by four consecutive ether extractions. Inositol phosphate analysis by MDD-HPLC (Mayr 1988) was carried out as described by Hatzack et al. (2001).

RT-PCR analysis

Total RNA was extracted from 1 g of frozen shoots from 5 day-old seedlings or of maturing kernels (20 days after pollination) of *lpa241* homozygous in the ACR background and wild-type ACR using the method described by van Tunen et al. (1988). The reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect *MIPS1S* gene transcript. First-strand cDNA was synthesized with an oligo (dT) primer from total RNA extracted from shoots and kernels. The primer used was a 35-base oligonucleotide with 17 dT residues and an adapter (5′-GGGAATTCGTCGACAAGC-3′) (Frohman 1990). All RNA samples were treated with DNase (1 unit/μg) before cDNA synthesis. The different samples of cDNA were then diluted to obtain a uniform concentration. First-strand cDNA was used as a template for PCR amplification. Amplification reactions containing an aliquot of cDNA synthesized from 5 μg of total RNA, 1× Promega polymerase buffer, 2.5 mM of $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.1 μM of each primer and 1 unit of *Taq* DNA polymerase (Promega, Madison, Wis.) were performed in a final volume of 50 μl. After the first denaturation step (5 min at 94 °C), the reaction mix underwent 20 cycles of denaturation at 94 °C for 1.5 min and annealing at 62 °C for 1 min. Extension at 72 °C for 15 min was performed to complete the reaction. A set of primers specific for the *orange pericarp-1* (*orp-1*) gene, which encodes the β-subunit of tryptophan synthase (Wright et al. 1992), was used to standardize the concentration of the different samples. *orp-1* specific sequences were amplified using the following primers: 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 *orp-1* amplification and hybridization were done in order to obtain similar amplification signals in the different samples. To ensure that amplification reactions were within linear ranges, the reactions were carried out for 20 cycles. For mRNA detection of the *MIPS1S* gene under analysis, the following specific primer sets were used: Zm656 (upstream primer 5′-TATAGCAATGTGTGCGCTGG-3′), and the specific *MIPS1S* primers (Larson et al. 1999) Zm1580 (downstream primer 5′-GTTCCCTTCCAGCAGCTAAC-3′). The amplified products was 925 bp and the identity of the MIPSIS (Gene Bank accessions AF056326) product was confirmed by sequencing (cDNA was amplified by high-fidelity PCR, P*fu* polymerase; stratagene). PCR products were fractionated on 1.2% (w/v) agarose gels, transferred onto the Amersham Hybond-NT membrane, and hybridized. The *MIPS1S* and *orp-1* products were hybridized with the PCR fragment obtained by amplification using the same primers. The probes were ^{32}P-labeled using random hexamer primers (Feinberg and Volgelstein 1983).

Results

Isolation and phenotype characterization of the *lpa 241* mutant

In order to generate low phytic-acid mutants, a population of mutagenized maize plants was obtained by treating pollen with EMS (ethyl-methanesulfonate) prior to pollination (Neuffer et al. 1978). Since normal mature maize seeds contain a high amount of phytic phosphate and a low free phosphate pool, a screening for seeds containing a high level of free phosphate (HIP phenotype) provides a useful tool for *lpa* (low phytic acid) mutant detection (Rasmussen and Hatzack 1998; Raboy et al. 2000). Approximately 600 M2 families were examined. The screening was carried out on extracts from milled seeds by titration of free phosphate using the molybdate staining assay (Chen et al. 1956). Extracts from putative mutants were then analyzed by a thin-layer Chromatography (TLC) method allowing the simultaneous detection of free phosphate and phytate (data not shown). Twenty eight out of 29 originally isolated HIP families turned out to be lethal developmental mutants impaired either in embryo (18 embryo-specific mutants) or endosperm (10 defective

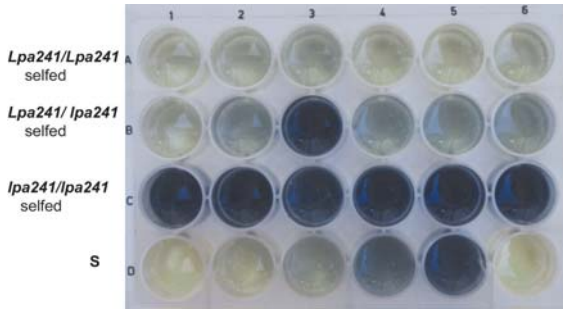

Fig. 2 Assay for free phosphate (HIP phenotype) in *lpa241* families. Single seeds from a given ear were crushed, extracted and assayed for free P using a microtitre plate-based colorimetric molybdenum staining assay. To allow for direct comparison, 100 mg of flour were extracted in 10 vol of HCl 0.4 N, and an equal aliquot vol was tested. The standards (*S*) contained 0, 0.20, 0.50, 1.50 and 3.0 μg of P

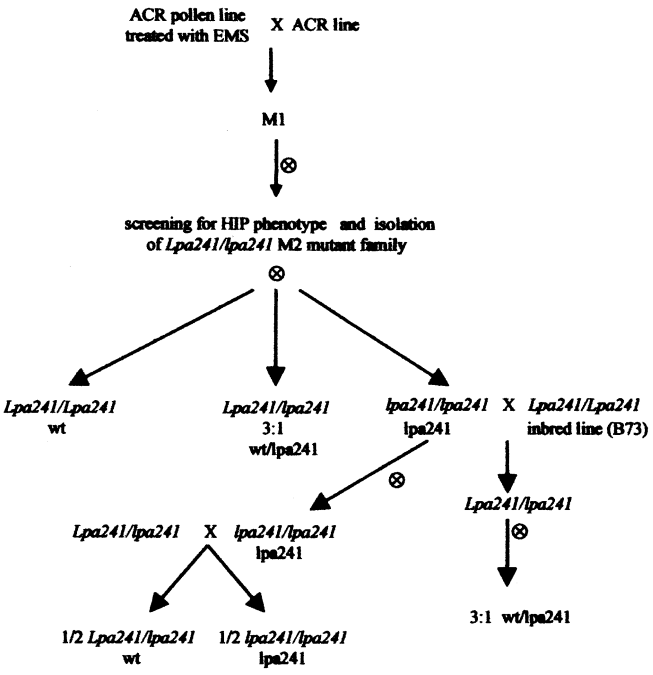

Fig. 3 Pedigree diagram. Genetic analysis shows the inheritance of the *lpa241* mutant as a monogenic recessive mutation

kernel mutants) development, thus showing P-related pleiotropic effects probably not associated with the phytic-acid pathway. However, one monogenic recessive non-lethal mutant that we named *lpa241* was isolated (Fig. 2), which germinated, developed and apparently reproduced normally (Pilu et al. 2002). The quantitative analysis of seed P fractions of this mutant compared to the parental line as a control, revealed an approximately ten-fold increase in the amount of free phosphate as well as a simultaneous dramatic decrease of about 90% in the phytic acid level, while no net change in seed total P occurred (Table 1). Heterozygous +/– seeds also exhibited a consistent increase in free phosphate (about three-fold) and a corresponding decrease in phytic acid content compared to the wild-type, indicating that the *lpa241* mutant allele may not be strictly recessive. An important feature of the *lpa241* mutant phenotype is that it does not appear associated to a significant reduction in seed dry weight in the original ACR background (Table 1). However, a remarkable reduction (around 30%) in the germination rate of homozygous mutant seeds (due also to the occurrence of defective seedlings) was observed (data not shown).

Genetic analysis

The genetic analysis of *lpa241* was based on the ascertainment of the presence or absence of the HIP phenotype (Fig. 2). About 15% of the seeds belonging to several segregant families were also tested by TLC analysis in order to confirm the correspondence between the lpa and HIP phenotype. The 3:1 segregation ratio of the *lpa241* mutant, observed in the F_2 generation of the initial cross, indicated a monogenic recessive defect. In order to confirm this finding, the original F_2 family was selfed, and the F_3 families obtained were analyzed. The F_3 progeny again showed a 3:1 segregation ratio and led to the establishment of two lines yielding only *Lpa241* or *lpa241* mutants as a result of the fixation of *Lpa241* or *lpa241 loci* in the homozygous condition (Fig. 3). A 3:1 ratio was also observed in the selfed progeny of *lpa241* plants outcrossed to inbred line B73 male-parents. Moreover, test-cross analysis showed a 1:1 segregation ratio (Table 2). In summary, the above results are consistent with a monogenic recessive basis of the *lpa* trait.

In order to establish the chromosomal-arm location of the *lpa241* locus, heterozygous *Lpa241/lpa24l* females were crossed with heterozygous or hyperploid B-A translocation males. The F_1 uncovering the HIP mutant phenotype was that obtained from crosses with the TB-1S

Table 1 Mature dry seeds of the indicated genotypes were weighed and assayed for seed total P, free P and phytic acid P. These fractions are expressed as P concentrations (atomic wt = 31) to facilitate comparison

Genotype	Seed dry weight[a] mg	Total P[b] mg g^{-1}	Pi free[b] mg g^{-1}	Phytic acid P[b] mg g^{-1}
+/+	184 ± 11	4.5 ± 0.42	0.3 ± 0.15	3.7 ± 0.21
lpa241/+	189 ± 5	4.4 ± 0.34	0.7 ± 0.18	3.3 ± 0.28
lpa241/lpa241	179 ± 6	4.6 ± 0.47	3.3 ± 0.40	0.4 ± 0.32

[a] Values are the mean ± SD of almost 60 seeds
[b] Values are the mean ± SD of seven replicates

Table 2 Segregation of *lpa241*/*lpa241* phenotypes observed in the F_2 obtained by selfing and test-cross progenies

Coded#	Genetic test:	Segregation			
		wt	Mutant	χ^2-value	*p*
R47(241)	F_2	29	6	1.15	0.3–0.2
R351(4)	F_2	98	31	0.49	0.5–0.3
R351(3)	F_2	60	19	0.04	0.9–0.7
R396-1	F_2	49	10	2.03	0.2–0.1
R395/R394-6	Test-cross	28	23	0.49	0.5–0.3

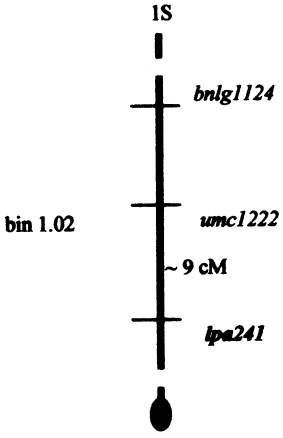

Fig. 4 Mapping of the *lpa241* locus. Approximate distance of *lpa241* from umc 1222 is shown on chromosome 1S

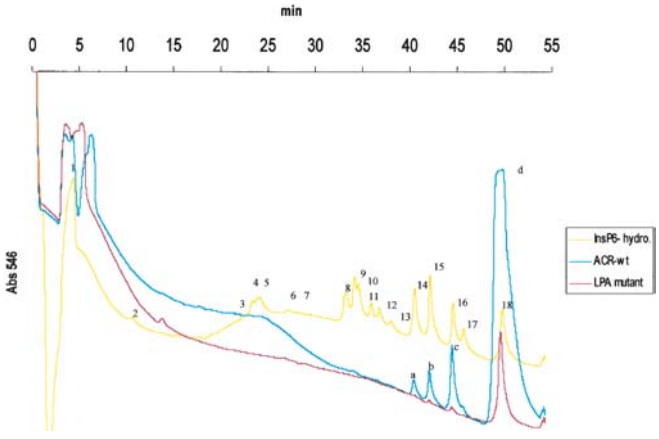

Fig. 5 MDD-HPLC analysis of inositol phosphates from the *lpa241* mutant and parent line kernels. TCA extract (100 μl each) from the *lpa* mutant and ACR parental line kernels were resolved on a Mono Q HR 5/20 column using a strongly acidic HCl gradient. Inositol phosphates were detected by MDD, a post-column detection procedure in which the organic dye PAR is mixed with the eluent containing Y^{3+}. The complex formation between Y^{3+} and PAR, resulting in a steady absorbance, is measured at 546 nm. Inositol phosphates and P-free outcompete PAR from the Y^{3+}-PAR complexes, yielding free PAR, which has a lower absorption than Y^{3+}-PAR. For better comparison, HPLC profiles were mirrored and stacked. Peaks in the InsP6 hydrolizate trace correspond to: *1*, InsP and Pi; *2*, Ins(1,4)P2; *3*, Ins(1,3,5)P3; *4*, Ins (1,3,4)P3; *5*, Ins(1,4,5)P3; *6*, Ins(1,5,6)P3; *7*, Ins(4,5,6)P3; *8*, Ins(1,2,3,5)P4; *9*, Ins(1,2,3,4)P4; *10*, Ins(1,2,4,5)P4; *11*, Ins(1,3,4,5)P4; *12*, Ins (1,2,5,6)P4; *13*, Ins(2,4,5,6)P4; *14*, Ins(1,4,5,6)P4/Ins(1,2,3,4,6)P5; *15*, Ins(1,2,3,4,5)P5; *16*, Ins(1,2,4,5,6)P5; *17*, Ins(1,3,4,5,6)P5; *18*, InsP6. The elution order listed is according to Hatzack et al. (2001)

male parent, thus indicating that *lpa241* lies on the short arm of chromosome 1S. A more refined position for *lpa241* was achieved by the analysis of simple sequence repeat (SSR) marker-distribution in a F_2 segregating population consisting of 54 seeds. A polymorphism for the markers umc 1,222 and bnlg 1124 established the position of the mutation on bin 1.02, at a distance of about 9 cM from umc 1,222 (Fig. 4). These values are similar to those reported for *lpa-1* Raboy's mutant (Raboy et al. 2000), suggesting the allelism of the two mutants.

Biochemical characterization by MDD-HPLC analysis

To gain information about the biochemical consequences of the *lpa241* mutation, we prepared extracts in tri-chloro-acetic acid from *lpa241* and wild-type mature seeds, and analyzed them by MDD-HPLC, a technique allowing the resolution and quantitative analysis of both phytic acid and its hypophosphorylated intermediates (Hatzack et al. 2001). The obtained results confirmed that in *lpa241* seeds the reduction of total InsPs is primarily accounted for by a very large reduction in phytic acid P (peak d) (Fig. 5), while no accumulation of other Ins phosphates is observable; this confirms the results previously obtained by TLC and suggests that *lpa241* is a mutation affecting the first step of the phytic acid pathway, namely the reaction catalyzed by MIPS. It is of interest that the MDD-HPLC analysis of wild-type seeds also allowed us to detect small amounts of InsP4 and P5: Ins(1,4,5,6)P4/Ins(1,2,3,4,6)P5 (peak a); Ins(1,2,3,4,5)P5 (peak b); Ins(1,2,4,5,6)P5 (peak c), besides an unknown peak with an elution time of approximately 5 min. The 60-min elution profile did not allow us to distinguish among the different InsP1 expected to elute early, and further investigations are needed for a final identification.

Expression of the *MIPS1S* gene in *lpa241* shoot and kernels

On the basis of the genetic and biochemical data presented above, both indicating a lesion in a *MIPS* gene, we verified the expression level of the *MIPS1S* gene during germination (5-day old seedlings) as well as during maturation of the kernels (20 days after pollination). For this purpose we carried out a RT-PCR analysis using

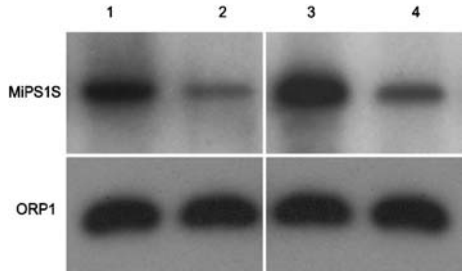

Fig. 6 RT-PCR analysis showing that expression of the *MIPS1S* gene in the *lpa241* mutant (*lane 2*, shoot; *lane 4*, kernel) is remarkably weaker than in wild-type (*lane 1*, shoot; *lane 3*, kernel). cDNAs were prepared using 3 μg of total RNA, extracted from shoot apices or maturing kernels (SuperScript First-Strand Synthesis System for RT-PCR). Amplification was made using specific primers for *MIPS1S* (Zm 656 + Zm 1580). The amplification band corresponding to the *MIPS1S* transcript is 925 bp, and *orp-1* was used as a control

specific primers for the *MIPS1S* sequence. Gene-specific PCR amplification of the cDNA-1S *MIPS* gene (*MIPS1S*) was obtained using the Zm 656 + Zm1580 specific primer pair (see Materials and methods). The Zm1580 primer was designed in a 3′ UTR sequence that was specific for *MIPS1S* (Larson and Raboy 1999). The amplified product was 925-bp long and the identity with the *MIPS1S* (Gene Bank accessions AF056326) gene was confirmed by sequencing (data not shown).

Results, presented in Fig. 6, show that: (1) *MIPS1S* appears to be expressed not only in maturing kernels, but also in shoot apices; (2) both in shoot and in kernels tissues the specific expression of *MIPS1S* gene is remarkably weaker in the case of the *lpa241* mutant than in wt, pointing to a mutation affecting the *MIPS1S* gene expression.

Discussion

Due to the nutritional and environmental problems connected with the presence of phytic acid in grains used for animals and human feeding (Cromwell and Coffey 1991), the "low phytic acid" trait represents a goal for the genetic improvement of maize and other crops used as fodder grain.

In a cereal non-mutant seed, phytic-acid P typically represents about 75%, and inorganic P about 5%, of seed total P (Lott et al. 2000). In the *lpa* mutants isolated in the last few years in maize (*Zea mays* L.) barley (*Hordeum vulgare* L.), and rice (*Oryza sativa* L.), such ratios are altered: mutant seeds have normal levels of total P, but greatly reduced levels of phytic-acid P (Raboy and Gerbasi 1996; Larson et al. 1998, 2000; Rasmussen and Hatzack 1998; Raboy et al. 2000).

So far, in both barley and maize, only two *lpa* loci have been identified: *lpa1* and *lpa2*. It has been hypothesized that *lpa 1-1* is a maize mutation in the early Ins supply pathway, i.e. in a MIPS-encoding gene, and *lpa2-1* is a mutation in an inositol phosphate kinase gene (Raboy et al. 2000; Shi et al. 2003).

The results so far accumulated in the literature indicate that the maximal phytic acid reduction compatible with seed survival and germination, and with normal grain yield, appears to be 55–65% for maize, 70% for barley and 45% for rice (Raboy et al. 2001). Moreover, a number of nutritional studies on poultry, swine and fish, fed with *lpa* maize or barley, demonstrated that grain-P absorbed by these animals is remarkably higher in the case of mutants than of the wild-type, so that there is much-less need for P or phytase supplementation (Ertl et al. 1998; Sugiura et al. 1998; Spencer et al. 2000), and that the amount of waste P is reduced as well. Finally, it was also shown that iron and zinc retention is significantly higher in human subjects fed with meal prepared from *lpa* rather than wild-type maize (Mendoza et al. 1998; Adams et al. 2000).

A "low phytic-acid mutant" might also be of particular value for studying the physiological roles of phytic acid in seed protection from biotic and abiotic stresses, as well as to transfer the trait to other plants producing edible seeds. In maize, the only two mutations causing very high phytate reductions (up to 95%) so far isolated, are lethal as homozygous (Raboy et al. 2001).

In the present work, following chemical mutagenesis, we have isolated a recessive maize mutant, named *lpa241*, with a large reduction (about 90%) in grain phytic acid and a quite relevant increase (about 10-fold) in grain-free phosphate content. The germination rate of mutant seeds was affected by about 30%; however, no other pleiotropic negative effects were evident for plant developement and seed maturation (Table 1). Homozygous mutant seeds appear in fact similar to wild-type in kernel-weight and phytase expression-extent during germination (data not shown).

The genetic analysis of the *lpa241* mutant based on the presence or absence of HIP phenotype (Figs. 2, 3) showed that the mutation is inherited as a monogenic recessive trait (Table 2). Nevertheless, the heterozygote had some observable effect on phytic-acid P and free Pi levels (Table 1). This might indicate that one single-functional gene copy is not sufficient to warrant the wild phenotype, but further data need to be collected in order to confirm this point. Mapping of *lpa241* carried out by using TB-A stocks and SSR molecular markers allowed the positioning of the gene on the chromosome 1S, bin 1.02, about 9 cM from umc 1,222 (Fig. 3). This is approximately the same position attributed by Raboy and co-workers to the *lpa1* mutant (Raboy et al. 2000), supporting the idea that *lpa241* may be an allele of the *lpa1* mutant. A complementation test between the two mutants should definitely ascertain this point. Analysis by "metal-dye" detection HPLC (MDD-HPLC) showed that seeds from the *lpa241* mutant contained low or undetectable levels of phytate, and no trace of hypophosphorylated inositol phosphate (Fig. 5). These data suggest that the *lpa241* mutant should be assigned to the *lpa1*, and not to the *lpa2*, class of *lpa* mutants (Raboy et al. 2000). Accordingly, the mutation

should regard MIPS, the first enzyme of the pathway, catalyzing the production of *myo*-inositol-3-phosphate from glucose-1-phosphate.

Even though no direct demonstration attributing the *lpa1-1* to the MIPS gene is at present available, the mutant's map position suggests identity of the *MIPS1S* sequence with the *lpa1* locus.

When by RT-PCR we compared the *MIPS1S* specific expression-extent in wild-type seedlings and maturing kernels, we obtained signal in both tissues (Fig. 6, lanes 1–3) indicating a lack of seed-specific expression. Moreover, a lower signal was registered for the *lpa241* mutant (Fig. 6, lanes 2–4) than for the wild-type, showing that the mutation affects MIPS1S gene-expression.

The identity of the *MIPS1S* cDNA amplified-products obtained were confirmed by sequencing, and BLAST database search was performed with the 3′ 925-bp amplified product and the predicted protein sequence (Altschul et al. 1997). This search revealed a 100% identity to *MIPS1S* (Gene Bank accessions AF056326) at the amino-acid level. More probably, it is of significance that we were never able to amplify the 5′ *MIPS1S* region from mutant cDNA using primers designed in the 5′ UTR region (data not shown). The mutation might in fact cause a drop in transcription levels or a transcript truncation, or else an incorrect transcript processing.

On the whole, these data indicate that the *lpa* mutant we isolated bears a lesion in a member of the *MIPS* gene family, *MIPS1S*, and represent the first direct molecular demonstration of the relationship between the *MIPS1S* gene-expression and the phytic acid accumulation in the maize kernel. Moreover, these results reveal that, despite maize has several closely related *MIPS* genes, these cannot substitute for each other, or they are under different developmental or temporal control. This is not surprising since the MIPS reaction (followed by the *myo*-inositol-3-phosphate phosphatase reaction) represents the sole de novo biosynthetic route to *myo*-inositol, a metabolite which plays a central role in several plant metabolic processes other than those regarding phytic acid, such as the synthesis of cell-wall components, carbohydrates belonging to the raffinose family, polyols, indole esters (conjugates with auxin), phytosphingolipids, phosphoinositides involved in membranes biogenesis, as well as the second-messenger Ins(1,4,5)P3, an important signal-transducter in both animal and plant cells (Loewus and Murthy 2000). Such multifunctionality may then justify either multiple genes or any relevant difference in the expression patterns of the MIPS coding genes.

At present, an analysis of the sequence of the MIPS1S genomic region is in progress in order to identify the molecular lesion related to the *lpa241* mutation. Moreover, we are introgressing the mutation in several inbred lines with the aim of verifying the penetrance and expressivity of the mutation, as well as of trying to avoid the negative pleiotropic effects on the germination rate.

Acknowledgements This work was supported by the Ministero dell'Universita e della Ricerca Scientifica e Tecnologica-Cofin 1998 (to G.G.). We wish to thank Mikael Blom Sorensen (Risoe National Laboratory, DK) for skilful assistance in MDD-HPLC experiments, and Dr. Anne Marie Lescure (Laboratoire de Biologie des Semences, INRA INA-PG, Versailles, F) for kindly providing phyS11 cDNA clones.

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