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Expression and Nucleotide Sequence of an INS (3) P_1 Synthase Gene Associated with Low-Phytate Kernels in Maize (*Zea mays* L.)†

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Most of the phosphorus (P) in maize (*Zea mays* L.) kernels is in the form of phytic acid. A low phytic acid (lpa) maize mutant, *lpa1-1*, displays levels reduced by 66%. A goal of genetic breeding is development of low phytic acid feedstocks to improve P nutrition of nonruminant animals and reduce the adverse environmental impacts of excess P in manure. The genetic basis of the *lpa1-1* mutation is not known, but previous genetic mapping has shown both the mutant phenotype and the Ins (3) P_1 synthase (MIPS) gene, which encodes the first enzyme, *myo*-inositol phosphate synthase, in the phytic acid biosynthetic pathway, map to the same chromosomal region in maize. Research was conducted to determine the expression of the MIPS gene in *lpa1-1* and wild-type kernels with similar genetic backgrounds and to ascertain if variation in the MIPS coding sequence could be inferred to be the basis of the mutation. MIPS enzyme activity determined by TLC was reduced 2−3-fold in mutant kernels. RT-PCR-based experiments using primers specific for the 1S-MIPS sequence indicated gene expression was reduced 50−60% in the mutant. Sequence analysis of the MIPS genomic sequence revealed 10 exons and 9 introns that are identical in both mutant and wild-type developing kernels. These findings support an association between reduced MIPS gene activity and low phytic acid content, but additional research should examine the promoter, the 5′UTR, or transcriptional controlling elements of the MIPS gene to ascertain the possible presence of a genetic lesion in those regions.

KEYWORDS: ***Zea mays* L.; gene activity and sequence analysis; low phytic acid; Ins (3) P_1 synthase**

INTRODUCTION

Developing seeds store phosphorus (P) and other minerals, the mobilization of which during germination provides the mineral nutrients essential for optimal early seedling growth. The storage and utilization of P during seed development and germination primarily involve the synthesis, deposition, and mobilization of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate or Ins P_6). Phytic acid is the most abundant P-containing compound in mature seeds (*1*). Seeds typically contain enough total P to satisfy a significant portion of ruminant animals' nutritional needs. However, nonruminants lack the ability to utilize the P stored as phytic acid (*2*). Phytic acid binds mineral cations such as Ca, Mg, Fe, Zn, Cu, and Co in the nonruminants' intestinal tract, rendering them unavailable (*3*, *4*). Seed-derived dietary phytic acid excreted in animal waste is a water pollutant (*5*). Phytic acid also has a negative impact on mineral nutrition in humans. Iron deficiency, which is considered to be a widespread dietary problem in the developing world, is believed to be caused by phytic acid binding to the mineral and forming complexes, thereby reducing its bioavailability (*6*).

Genetic approaches have been undertaken to address the phytic acid problem described above. Researchers have developed low phytic acid (lpa) maize mutants (*lpa1-1*, *lpa2-1*, etc.) using the mutagen ethyl methane sulfonate (EMS) (*7*, *8*). When screening for EMS-induced lpa mutations in maize, Raboy et al. (*8*) found two types of biochemical phenotypes, *lpa1-1* and *lpa2-1*. The designations *lpa1-1* and *lpa2-1* indicate that they are the first recessive, mutant alleles of the *lpa1* and *lpa2* loci, respectively (*9*). The most common type, *lpa1* mutants, exhibit large reductions of phytic acid P and corresponding increases of P_i. The *lpa1-1* mutant has the potential of alleviating environmental and nutritional problems associated with grain phytic acid (*10*). The *lpa2-1* mutants show large reductions of

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† The nucleotide and peptide sequences reported in this paper have been submitted to GenBank under the accession numbers AF323175 and AAG40328, respectively.

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phytic acid P, but only a small increase in P_i, as compared to the *lpa1-1* mutation. The *lpa2-1* mutation is also different from the *lpa1-1* mutation in terms of the increased level of "lower" inositol polyphosphates.

The biosynthetic pathway of phytic acid consists of two phases: the production of Ins and its subsequent polyphosphorylation. The first committed step of Ins biosynthesis involves Ins (3) P_1 synthase (formerly known as *myo*-inositol phosphate synthase or MIPS) that converts Glc-6-P to Ins (3) P_1. This conversion provides the only de novo source of the Ins ring in all organisms (*11*). Because Ins is the first substrate in the phytic acid biosynthetic pathway, a mutation in the MIPS gene could result in a reduction in phytic acid biosynthesis and an accompanied increase in inorganic phosphorus (P_i) content as found in the *lpa1-1* mutant. A mutation in any other gene further down the pathway would not result in an accompanied increase in P_i (*12*).

The *lpa2* mutant has been shown to be a result of a mutation in the inositol phosphate kinase gene that affects the phytic acid biosynthesis pathway downstream of the Ins biosynthesis (*13*). We hypothesized that the *lpa1-1* mutant phenotype was the result of a genetic change in the MIPS encoding region that subsequently reduced the expression of the gene and the enzymatic activity of MIPS.

Bulk-segregant analysis of an F_2 population derived from a wild-type × *lpa1-1* cross established linkage of *lpa1-1* to the restricted fragment length polymorphism (RFLP) marker, *umc157*, which maps to the distal portion of chromosome 1S (*8*). Further studies of recombinant individuals placed *lpa1-1* ~7.7 cM proximal to *umc157*. Mapping results indicate that the maize genome has up to seven MIPS loci (*12*). One complete maize MIPS cDNA has been sequenced (Gene Bank accession AF056326). This MIPS gene maps to the same location on the maize 1S chromosome as the *lpa1-1* mutation. These results support the hypothesis that the 1S chromosome MIPS gene is a candidate gene for the *lpa1-1* mutation (*12*).

Many of the biochemical and physiological aspects of the MIPS gene are known. The *INO1* locus was first identified as the structural gene for MIPS in yeast, *Saccharomyces cerevisiae* (*14*). Since that report, various homologues of this gene have been identified in different plant species (*15*). The first plant gene to be characterized was from the aquatic angiosperm, *Spirodella polyrrhiza*. The gene was shown to be rapidly and spatially up-regulated during an ABA-induced morphogenic response (*16*). In 1999, Yoshida et al. for the first time showed the relationship between the expression of MIPS and phytin synthesis in developing rice (*Oryza sativa* L.) kernels (*17*).

The objectives of this study were to determine if the changes in the MIPS gene expression and enzymatic activity in developing *lpa1-1* maize kernels were consistent with the phenotype conferred by the mutation. RT-PCR and TLC autoradiography were used to study differences in MIPS expression and activity. We also sequenced the entire genomic DNA of the 1S MIPS gene in the closely related mutant *lpa1-1* and wild-type genotypes to identify a lesion that might be associated with the altered gene expression.

MATERIALS AND METHODS

Plant Materials. Seeds were obtained through controlled pollination of partially inbred backcross-one (BC_1) progenies displaying wild-type and *lpa1-1* mutant phenotypes. Wild-type and mutant BC_1 progeny pairs were obtained in two recurrent parent backgrounds, A632 and B73. Seeds of wild-type and *lpa1-1* mutant inbred progenies developed by Dr. Victor Raboy (USDA-ARS, Aberdeen, ID) were provided by FFR Cooperative (West Lafayette, IN). The plants were grown in the field (Waterman Research Farm, Columbus, OH) and were self-pollinated through controlled procedures. The kernels were collected 26−30 days after silking, corresponding to the R4/R5 stage of development (*18*). The texture of the kernels was doughy, indicating they had reached physiological maturity. After collection, the kernels were immediately frozen in liquid nitrogen.

DNA and RNA Isolation. Genomic DNA was isolated from 4-week-old leaves of each of the four genotypes using the Plant DNAzol reagent and manufacturer's protocol (Gibco BRL Life Technologies, Rockville, MD).

Total RNA was extracted from the leaf samples and developing kernels using the lithium chloride precipitation method. The plant material (1 g fresh weight) was ground in liquid nitrogen with a sterile mortar and pestle. RNA extraction buffer [100 mM LiCl, 1% (w/v) SDS, 100 mM Tris-HCl, pH 9.0, 10 mM EDTA] was added (5 mL), and the material was transferred to a sterile tube. Cold phenol/chloroform/isoamyl alcohol (25:24:1) (Gibco BRL Life Technologies) was added (4 mL), and after 5 min of shaking, the tubes were centrifuged at 10000*g* (Beckman J2-21 centrifuge, Fullerton, CA) for 20 min at 4 °C. To the aqueous phase was added an equal volume of chloroform, which was mixed by gentle inversion followed by centrifugation at 10000*g* for 5 min at 4 °C. Additional LiCl was added to the supernatant to increase the final concentration to 2 M. Samples were stored overnight at 4 °C to precipitate the RNA. The samples were then centrifuged at 10000*g* for 30 min at 4 °C to pellet the RNA. The supernatant was poured off, and cold RNase-free water (400 μL) was added to dissolve the pellet. The solution was transferred to a 1.5 mL microcentrifuge tube. Ethanol precipitation was performed by adding 100% (v/v) ethanol (800 μL) and 2 M sodium acetate (40 μL). The tubes were shaken gently for 30 s and then stored at 20 °C for 3 h followed by centrifugation at 10000*g* for 20 min at 4 °C. The resulting RNA pellet was washed with 70% (v/v) ethanol and air-dried before being redissolved in RNase-free water (100 μL). Aliquots of RNA were stored at −80 °C after quantification in a spectrophotometer (Beckman DU-50) using optical density (OD) readings at 260 nm.

cDNA Synthesis and RT-PCR. First-strand cDNA synthesis was performed using the Ready-To-Go T-Primed First-Strand Kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and 5 μg of total RNA. The RT-PCR (25 μL) was conducted with different aliquot sizes of first-strand cDNA, 10× PCR buffer (2.5 μL) (Perkin-Elmer, Cetus, Norwalk, CT), $MgCl_2$ (2 mM), dNTP (200 μM), Taq DNA polymerase (1 unit), 1S-MIPS gene specific primers (forward, ZmF 3160, GCTCTTGGCTGAGCTCAGCA and reverse, ZmGSPR 3599, GTTCCCTTCCAGCAGCTAAC) (0.2 μM each) (*12*). The amplification reaction was performed with a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer Cetus) using 30 cycles of 40 s at 94 °C, 40 s at 58 °C, and 1 min at 72 °C. The products were analyzed on 1% (w/v) agarose gels stained with ethidium bromide. Primers of the actin gene (forward, GGACTCTGGTGATGGTG, and reverse, ACCTTAATCTTCATGCTGC) were used in positive control reactions. A negative control with no template DNA was also included for each set of PCR reactions.

Determination of the MIPS Enzyme Activity. The activity of the MIPS enzyme in the developing kernels was determined by TLC. D-[1-^{14}C]Glc-6-P and *myo*-[U-^{14}C]inositol were used as standards (NEN Life Science Products, Boston, MA). A crude enzyme extract was prepared from the developing maize kernels of the four genotypes using the procedure of Barnett et al. (*19*). Kernels (1 g of fresh weight) were ground in liquid nitrogen and mixed with 0.3 mL of extraction buffer [100 mM Tris acetate, 14 mM NH_4Cl, 60 μL of plant protease inhibitor (Sigma, St. Louis, MO)]. The homogenate was centrifuged at 13000*g* for 20 min at 4 °C. Protein concentration was estimated with the BCA Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard. The supernatant (5 μL) containing ~100 μg of protein was resuspended in 20 μL total volume of assay mixture (100 mM Tris acetate, 14 mM NH_4Cl, 0.8 mM NAD^+, and 1 mM D-[1-^{14}C]Glc-6-P). During the 1-h incubation at 30 °C, the labeled substrate D-[1-^{14}C]Glc-6-P was converted to [^{14}C]inositol-1-P by MIPS in the plant extract. The reaction was stopped by boiling for 3 min. Calf intestinal alkaline phosphatase (CIAP) (1 unit) (Gibco BRL Life Technologies) was added, and the reaction was incubated at 37 °C for 30 min. During this time, [^{14}C]inositol-1-P was dephosphorylated by

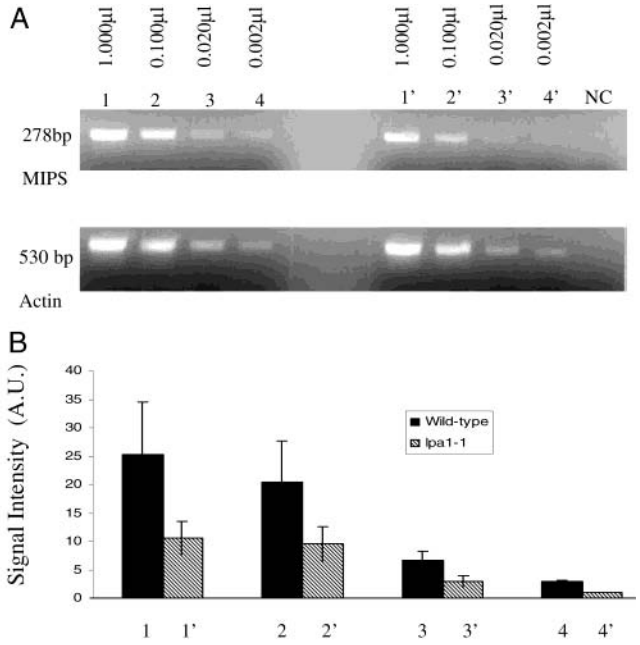

Figure 1. Detection of MIPS gene expression in maize kernels of A632 wild-type and A632 *lpa1-1* by RT-PCR. (**A**) RT-PCR was performed with the chromosome 1S specific MIPS gene using a series of dilutions (1, 0.1, 0.02, and 0.002 μL) of first-strand cDNA from kernels of maize lines A632 (lanes 1–4) and A632 *lpa1-1* (lanes 1′–4′). NC was the negative control. Expression of the housekeeping actin gene was used as positive control. (**B**) Mean signal intensities of MIPS expression in kernels from two separate RT-PCR experiments are shown: (solid bars) 1–4 wild-type; (slashed bars) 1′–4′ *lpa1-1*. The values are relative to the lowest signal (4′) taken as 1. Error bars represent SD based on two different measurements.

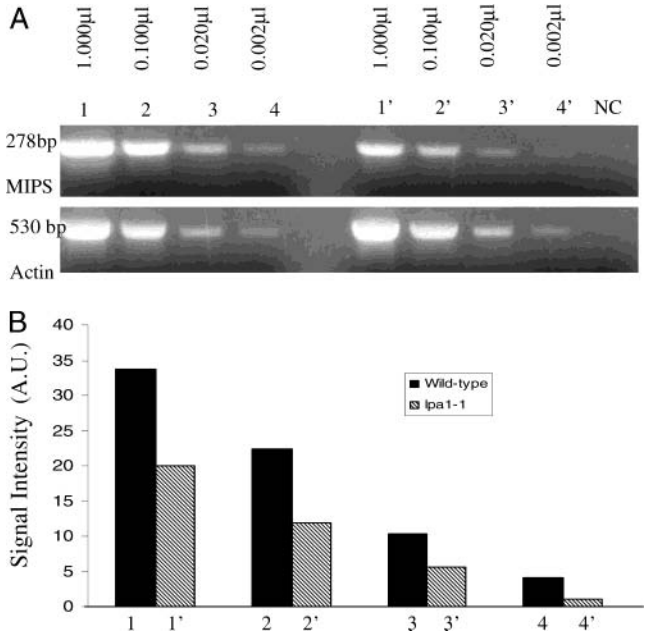

Figure 2. Detection of MIPS gene expression in maize kernels of B73 wild-type and B73 *lpa1-1* by RT-PCR. (**A**) RT-PCR was performed with the chromosome 1S specific MIPS gene using a series of dilutions (1, 0.1, 0.02, and 0.002 μL) of first-strand cDNA from kernels of maize lines B73 (lanes 1–4) and B73 *lpa1-1* (lanes 1′–4′). NC was the negative control. Expression of the housekeeping actin gene was used as positive control. (**B**) Signal intensities of MIPS expression in kernels are shown: (black bars) 1–4 wild-type; (slashed bars) 1′–4′ *lpa1-1*. The values are relative to the lowest signal (4′) taken as 1.

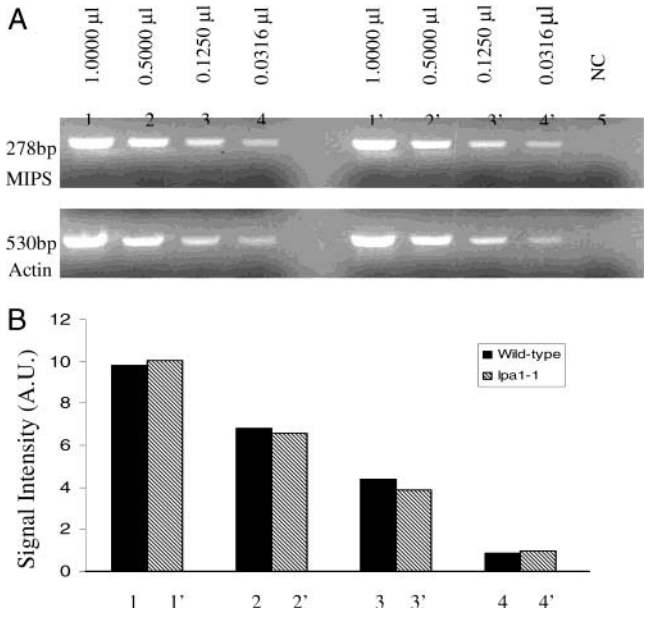

Figure 3. Detection of MIPS gene expression in leaves by RT-PCR. (**A**) RT-PCR was performed with the chromosome 1S specific MIPS gene primers using a series of dilutions (1, 0.5, 0.125, and 0.0316 μL) of first-strand c-DNA from leaves of maize inbred lines A632 (lanes 1–4) and A632 *lpa1-1* (lanes 1′–4′). Lane 5 was the negative control. Expression of the housekeeping actin gene was used as positive control. (**B**) Signal intensities of MIPS expression in leaves are shown: (black bars) 1–4 wild-type; (slashed bars) 1′–4′ *lpa1-1*. The values are relative to the signal from 4′ taken as 1.

CIAP to produce [^{14}C]inositol. The reaction was stopped by boiling, and the extract was subsequently clarified by centrifugation. The radioactive products of the enzyme assay were separated on flexible silica gel plates (Whatman, Clifton, NJ) by two-dimensional TLC using *n*-propanol/pyridine/water (3:1:1) in the first dimension and *n*-butanol/acetic acid/diethyl ether/water (9:6:3:1) in the second dimension (*16*). The TLC plates were subjected to autoradiography for 7 days, and the radioactivity was quantified using a phosphor-imager (PhosphorImager SI, Molecular Dynamics, Sunnyvale, CA). *myo*-[U-^{14}C]inositol was used as a standard in addition to the standard protocol in one of the reactions to determine the migration of inositol in the TLC phases.

Sequencing of the 1S-Chromosome MIPS Gene. Sequencing of the MIPS gene was accomplished using four separate cloning experiments. Genomic DNA extracted from leaves of the A632 and B73 pairs of wild-type and *lpa1-1* mutant inbred progenies was used to amplify the MIPS gene. The 3′-primer specific for the 1S-chromosome MIPS gene, ZmGSPR 3504, GTTCCCTTCCAGCAGCTAAC, (*12*) was used together with a 5′-primer, ZmF 2726, TCAAGCATTCAGGTCCAAGG, designed from the maize MIPS cDNA sequence information (GenBank accession AF056326) to amplify a fragment of the MIPS gene from genomic DNA. PCR was performed using genomic DNA (90 ng) and the same conditions as previously described for the RT-PCR. The PCR products were separated by agarose gel electrophoresis, and the band of interest was purified using the QIAquick Gel Purification kit (Qiagen, Valencia, CA). The purified PCR products were quantified with a spectrophotometer as described. The amplified products were cloned into a TA vector using the procedure described by the manufacturer (Invitrogen, San Diego, CA). Several white colonies were chosen for sequencing. An individual white colony was transferred to LB medium (5 mL) containing ampicillin and cultured overnight at 37 °C. The plasmid DNA was isolated using QIAprep Spin Miniprep Protocol (Qiagen). An amplification reaction with the purified DNA (90 ng) and plasmid specific T7F (TAATACGACTCACTATAGGG) and M13R (GGAAACAGCTATGACCATG) primers was performed using the same conditions as described. The PCR product was analyzed on a 1% (w/v) agarose gel to check for correct insert size of the plasmid. Only the amplification products with the

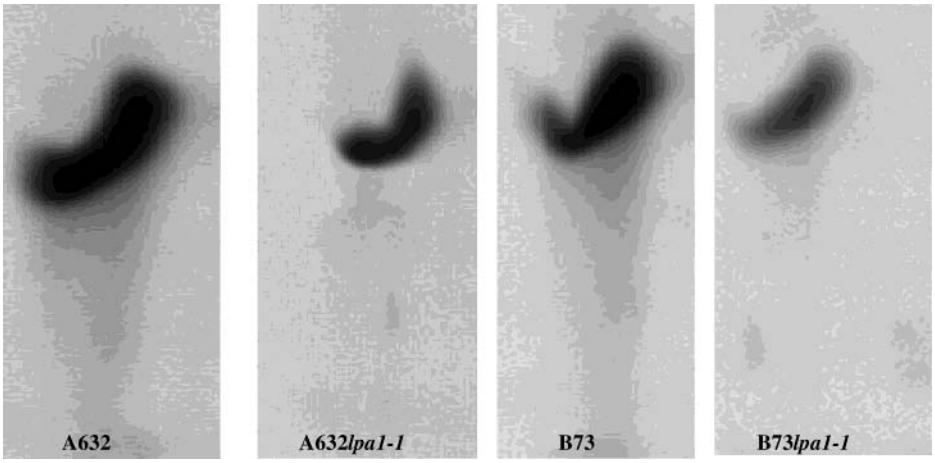

Figure 4. TLC assay of MIPS enzyme activity in mazie kernels. Crude protein extracts from kernels of maize inbred NIL pairs A632 and A632 *lpa1-1* and B73 and B73 *lpa1-1* were assayed for MIPS enzyme activity using D-[1-^{14}C]Glc-6-P as substrate. *myo*-[U-^{14}C]inositol was used as standard. The products of the reaction were separated by two-dimensional TLC and visualized by autoradiography. A portion of the radiograph containing labeled inositol is shown.

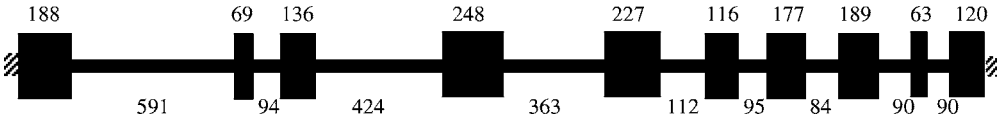

Figure 5. Schematic diagram of MIPS gene depicting 10 exons (black boxes) and 9 introns (solid line). The 5′ and 3′UTR are shown in hatched bars. Sizes of exons are noted above the boxes in base pairs. Sizes of introns, in base pairs, are denoted below the solid lines.

correct insert size were used for sequencing reactions. One or two clones were used for sequencing the fragments. The cycle sequencing reaction was performed using the Big Dye sequencing kit (Perkin-Elmer, Cetus) and the T7F and M13R primers following the manufacturers' protocol. The PCR products were sequenced using the ABI Prism 310 (PE Applied Biosystems, Foster City, CA). New primers were designed using known intron sequences to continue with the sequencing process toward the 5′ end of the gene. The three other primer pairs used were ZmF 2202, AACGACACGATGGAGAATCT, and ZmR 2848, AACGAGGTGAAAGATCA GCT; ZmF 1016, ATCAGAGTCGGCAGCTACAA, and ZmR 2373, GGAACAGA AATGCAACACCA; and ZmF 95, AGCCTCCTTCCTCCTCTCAC, and ZmR 1523, TAATGTCCCAGCCTCCGAAC. The sequence obtained from each sequencing reaction was compared with the known cDNA (GenBank accession AF056326) using the BLAST 2 sequences tool provided in the NCBI web site (http://www2.ncbi.nlm.nih.gov/gorf/wblast2.cgi) (*20*).

In addition, direct sequencing of the gel-purified PCR product was performed at the Molecular and Cellular Imaging Center (MCIC), OARDC, Wooster, OH, using the same primers as described above.

RESULTS AND DISCUSSION

Differential Expression of the MIPS Gene in Mutant and Wild-Type Kernels. RT-PCR was used to detect the expression of the MIPS gene in developing kernels of the wild-type and *lpa1-1* mutants. When different aliquot sizes of first-strand MIPS cDNA were used as templates with 1S-MIPS gene specific primers for RT-PCR, a reduction in gene expression of the mutant A632 *lpa1-1* kernels compared to A632 kernels was observed (**Figure 1A**,**B**). Using RFLP analysis, Larson and Raboy (*12*) showed that the two primers amplify a PCR product from the chromosome 1S MIPS locus near *lpa1*. Signal intensity comparisons of the MIPS gene amplification products for lanes with equal amounts of MIPS template cDNA from A632 and A632 *lpa1-1* showed a ~2-fold or slightly greater reduction in the mutant versus wild-type kernels. Signal intensity of the actin gene amplification from the same template cDNA aliquot sizes showed no differences in mutant and wild-type kernels (**Figure 1A**). Similar results were obtained with RT-PCR of wild-type B73 and B73 *lpa1-1* kernels (**Figure 2**).

Expression of MIPS Gene in Mutant and Wild-Type Leaves. RT-PCR experiments were done to determine the expression levels of the 1S MIPS gene in leaves of the four genotypes studied. No difference in the level of MIPS gene expression was observed between mutant and wild-type leaves using the 1S-MIPS gene specific primers (**Figure 3A**,**B**). Expression of actin gene was also uniform across the genotypes as observed from the band intensity obtained by amplification with equal amounts of template (**Figure 3A**).

MIPS activity has been shown to have temporal and spatial regulation in rice (*17*). In our study, differences in MIPS transcript accumulation and enzyme activity were detected in kernels of *lpa1-1* mutants and wild-type. The RT-PCR results show that MIPS expression is not affected in leaves of the *lpa1-1* mutant plants, whereas differences in the level of MIPS expression in the leaves versus the kernels are observed.

The PCR primers used in this study have been shown to be specific to the 1S maize chromosome by RFLP analysis (*12*). However, the maize genome may have at least seven copies of the MIPS gene (*12*). Despite the specificity of the primers, it is possible that the results observed could have arisen from amplification of more than one copy of the MIPS gene.

Quantification of MIPS Enzyme Activity in Developing Maize Kernels. MIPS activity in wild-type and mutant kernels was determined by quantifying the amount of [^{14}C]-*myo*-inositol using two-dimensional TLC and autoradiography (**Figure 4**). The reaction product, labeled Ins (3) phosphate, was converted to free Ins by CIAP. *myo*-[U-^{14}C]inositol was run as a standard in parallel to identify the inositol migration in the TLC gel. A 2−3-fold reduction in [^{14}C]Ins signal strength was detected in developing kernels of the A632 *lpa1-1* mutant as compared to the A632 wild-type. The experiments were repeated twice across both the A632 *lpa1-1* and A632 and the B73 *lpa1-1* and B73 progeny pairs. Similar results were obtained each time, indicating that MIPS enzyme activity was reduced in *lpa1-1* mutant kernels compared to wild-type kernels.

Sequencing. Sequencing results showed that the MIPS genomic sequence contains 3618 bp (**Figure 5**) (GenBank entry AF323175) with 10 exons and 9 introns. The 5′UTR of the sequence is composed of the first 95 bp. The positions of the exons are as follows: exon 1, 96−283; exon 2, 875−943; exon 3, 1038−1173; exon 4, 1598−1845; exon 5, 2209−2435; exon 6, 2548−2663; exon 7, 2759−2935; exon 8, 3020−3208; exon 9, 3299−3361; and exon 10, 3452−3571. The 3′UTR is from 3572 to 3618. The translation product of the MIPS gene is composed of 510 amino acids (GenBank entry AAG40328). Sequence analysis of the MIPS gene in soybean (*Glycine max* L. Merr.) had revealed a single nucleotide change in the MIPS gene to be responsible for a change in seed phenotype of increased inorganic phosphate, decreased phytic acid, and a decrease in total raffinosaccharides (*21*). A similar lesion in the MIPS gene on the 1S chromosome in maize caused by the chemical mutagen EMS, used for the development of the *lpa1-1* mutant, was thought to be responsible for the reduction in phytic acid content. When the four sequences from the respective mutant and wild-type A632 and B73 genotypes were compared using the BLAST 2 sequences, no differences were detected between the entire nucleotide sequences. This result was unexpected considering the average frequency of random single-nucleotide polymorphism (SNP) in maize is 1 per 104 bp (*22*). According to our results, the 1S chromosome MIPS gene is highly conserved. However, the MIPS genomic sequence differed from the published cDNA sequence (GenBank accession AF056326) at positions 30, 36, 148, and 2825. A 12 bp insertion, from 66 to 77, found in our sequence was not present in the published cDNA sequence. Identical nucleotide sequencing results were also obtained from the direct sequencing of PCR products by the Molecular and Cellular Instrumentation Center of The Ohio Agricultural Research and Development Center, Wooster, OH.

We speculate that a mutation could have affected the promoter or a transcriptional factor controlling MIPS expression. Such a mutation may diminish or prevent binding of RNA polymerase to specific sites of the MIPS promoter and hence its transcription. Analysis of the 5′ upstream of the genomic sequence of the MIPS gene could have addressed the possibility of a mutation in the promoter region. In addition to promoter regulation, expression of eukaryotic genes can also be regulated by enhancers. These elements may be thousands of base pairs away from the transcription start site. Most enhancer elements function in a complete or partial tissue-specific manner; that is, they frequently enhance the transcription of genes in only specific target tissues (*23*). In the case of *lpa1-1*, there could be an enhancer element that is changed so that MIPS expression is reduced in mutant kernels.

In summary, our results indicate that there is a reduction in MIPS gene expression in *lpa1-1* mutant maize kernels. MIPS gene expression is both spatially and temporally regulated, and the differential expression between the *lpa1-1* mutant and wild-type genotypes appears not to be linked to a lesion in the MIPS coding sequence. It would seem to be caused by a change either in the promoter region or in a regulatory element located elsewhere in the genome. Future research on regulatory elements may shed more light on the molecular mechanism controlling the differential expression of the *lpa1-1* MIPS gene.

ABBREVIATIONS USED

P, phosphorus; lpa, low phytic acid; MIPS, Ins (3) P_1 synthase; EMS, ethyl methanesulfonate; RFLP, restricted fragment length polymorphism; OD, optical density; CIAP, calf intestinal alkaline phosphatase; SNP, single-nucleotide polymorphism; SD, standard deviation.

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