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Sequence analysis of MIPS-1s gene isolated from the low-phytic-acid inbred line of maize Qi319 and characterization of its expression patterns

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Abstract

Myo-inositol 1-phosphate synthase (MIPS) is a pivotal enzyme that initiates the first step of phytic acid synthesis. In the present study the *MIPS-1s* gene isolated was from an inbred line of maize Qi319 containing low contents of phytic acid (*lpa*). Its sequence and expression patterns were also characterized. Results showed that the full-length genomic DNA sequence of the *MIPS-1s* gene is comprised of ten exons and nine introns, with a length of 3596 bp, and an open reading frame of 1533 bp that encodes a protein with 510 amino acids. A single amino acid substitution was found, compared to the sequence of the inbred maize line B73. *In silico* promoter analysis revealed various cis-elements that might contribute to the differential regulation of *MIPS-1s*. The expression patterns of MIPS-1s proteins in leaves, roots, flowers and developing seeds of maize were also investigated using western blot. It showed similar patterns compared to the control line (Chang 7-2), suggesting that the MIPS-1s protein plays similar roles in the process of maize development and growth. Notably, it was found that the control line had a dramatically higher abundance of MIPS-1s protein than did Qi319, in the developing seeds and especially during the early period. This strongly indicates that higher levels of MIPS expression should precede and/or accompany synthesis and accumulation of phytate.

Key words: Maize, myo-inositol 1-phosphate synthase (MIPS), low phytic acid (LPA), western blot (WB), expression

Introduction

Worldwide, maize (*Zea mays* L.) is the feed ingredient used most commonly for livestock. Phosphorus (P) is an essential nutrient for all forms of life and in maize seeds it is stored primarily as phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate, InsP6), which is considered as the major form of phosphorus. About 90% of the phytic acid within maize seeds is deposited in the embryo, and the remaining 10% is stored in the aleurone layer (Shi et al. 2003). Phytic acid is degraded by phytase during germination, which provides mineral nutrients that are essential for seedling growth and development (Hegeman et al. 2001). However, because they lack the enzyme phytase, monogastric animals (e.g., swine, poultry, and fish) are unable to digest phytate efficiently. As a result, a large amount of phosphorus in undigested phytate cannot be absorbed, so it is then released in animal waste and hence contributes to water pollution (Bregitzer and Raboy 2006). Furthermore, the high phytic acid content of grains induces chelating effects upon mineral cations, leading to malnutrition of monogastric animals (Aluru et al. 2011). Breeding seeds to have reduced levels of phytic acid is, therefore, a desired target for the genetic improvement of major crops, including maize, rice, soybean, wheat, and barley. Over the past decade, various crops with low phytate genotypes have been identified — they exhibit low phytic acid content in their seeds, and improved bio-availability of Zn and Fe (Aluru et al. 2011). In maize, three types of low phytic acid mutations (*lpa1*, *lpa2* and *lpa3*) have been reported (Raboy et al. 2000; Shi et al. 2003; Shi et al. 2005; Shi et al. 2007).

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Phytic acid is synthesized during seed development and deposited in mature seeds. It is biosynthesized from glucose 6-P that is first catalyzed to myo-inositol 3-phosphate [Ins(3)P1] by myo-inositol 3-phosphate synthase, which in turn encodes MIPS, an enzyme that is essential in the first step of inositol phosphate biosynthesis. Yoshida et al. (1999) confirmed a close relationship between Ins(3)P1 formation and phytic acid biosynthesis in developing seeds. The MIPS-coding sequence has been cloned and characterized in some seed plants, such as *Zea mays* L. (Larson and Raboy 1999), *Oryza sativa* L.(Yoshida et al. 1999) and *Glycine max* L. (Hegeman et al. 2001), it turns out that the *MIPS* genes cloned from different plants are highly conserved at both the nucleotide and protein levels (Abid et al. 2009). In maize, seven homologous *MIPS* sequences on different chromosomes have been genetically mapped; the *lpa1* mutation was mapped at the position of the myo-inositol 1-phosphate synthase gene, which was located at the short arm of chromosome 1 (i.e., *MIPS-1s*) (Larson and Raboy 1999). Gene sequence analysis of *MIPS-1s* did not discover lesions within the coding sequence (Shukla et al. 2004), but the *MIPS* gene transcripts and its enzyme activity is 50-60% lower in *lpa1* seeds as compared to those of the wild type (Pilu et al. 2003; Shukla et al. 2004). A *MIPS* gene in rice, known as *RINO1*, is highly expressed in aleuronic layers of developing seeds and coinciding with phytic acid accumulation. A rice mutant with reduction in *RINO1* expression, obtained using an antisense approach with a seed-specific promoter, showed 70% reduction in phytic acid within the seed (Kuwano et al. 2009). In a soybean mutant, a single base change in the *MIPS* gene causes an amino acid replacement from lysine to asparagine, leading to a decrease of ~90% in the specific activity of the seed-expressed myo-inositol 1-phosphate synthase (Hitz et al. 2002). Moreover, soybean lines with a drastic reduction of phytate content have been obtained by partially silencing the *MIPS* gene using RNAi technology (Nunes et al. 2006). Because *MIPS* plays a critical role in phytic acid biosynthesis in the developing seed, alteration of the *MIPS* gene is a potential way to develop crops containing low phytic acid. However, little is known about the expression of MIPS protein.

Previously, we identified a recessive *lpa* mutation in a inbred line of maize named Qi319 which showed normal agronomic traits compared to other inbred lines, including Chang 7-2, Huang C and Zheng 58 (Gao et al. 2013). To understand the function of the *MIPS* gene and the mechanisms of phytic acid accumulation in Qi319 seeds, the genomic DNA of the *MIPS-1s* gene and its promoter in Qi319 and control genotypes were cloned and sequenced to identify possible lesion(s) in CDS or regulatory region that might be associated with altered gene expression. Additionally, western blot (WB) analysis was carried out to investigate potential changes in MIPS protein expression during seed development.

Materials and methods

Plant materials

This study was conducted in the research field of Hebei Agricultural University in Baoding, Hebei province, China during the years, 2009-2014. An inbred line of maize (*Z. mays* L.) called Qi319 was used as a low phytic acid line, and three lines with high phytic acid content were used as a control *viz*., B 73, Chang 7-2 and Chang 3154. All lines were grown in the field under regular cultivation conditions and they were self-pollinated using controlled procedures. Plant tissues were taken at different growth and developmental stages, leaves and roots were sampled at the seedling and anthesis stages, while tassels, pollen and ears were sampled at the flowering stage. At the flowering stage, each flower was tagged at anthesis, and the resulting ears were collected at 1, 2, 3, 7, 10, 15, 20, 25 and 30 days after fertilization (DAF). The sampled tissues were submerged into liquid nitrogen and then stored at –70°C until use.

DNA isolation and PCR amplification of the MIPS-1s gene and its promoter sequence

Genomic DNA was isolated from leaf tissues of the four inbred maize lines following established protocol (Zhang et al. 2013). Based on the the DNA sequence of GenBank accession AF 323175, three pairs of primer sets (MIPS-Frag1, MIPS-Frag2 and MIPS-Frag3) were designed (Table S1). The 5' end of the sequences of *MIPS-1s* was amplified with the primer set MIPS-Frag1, and the middle and 3' regions were amplified with primer sets MIPS-Frag2 and MIPS-Frag3, respectively. The three fragments were sequenced and assembled based on overlap regions using Sequencher software (Gene Codes Corp, Ann Arbor, MI, USA). All PCR amplifications were performed with a professional standard 96 Gradient thermocycler (Biometra) using 30 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. The PCR (total volume 20 μL) was conducted with different aliquots of genomic DNA as a template, 10×PCR buffer (2 μL), $MgCl_2$ (1.8 mM), dNTP (200

μM), *Taq* DNA polymerase (1 unit), and *MIPS-1s* gene-specific primers (0.2 μM each). The products were analyzed on 1% (w/v) agarose gels stained with ethidium bromide. A negative control without template DNA was also included for each set of PCR reactions. PCR fragments of the expected sizes were ligated into a pMD19-*T* vector and sequenced by Sangon Biotech (Shanghai) Co., Ltd.. The sequence obtained from each reaction was assembled using the software program Sequencher 4.1.4 (Gene Codes Corp, Ann Arbor, MI, USA).

The promoter region of *MIPS-1s* was amplified using 3'-primer D (AATGGAATGCGAAGCGAGG TAGCGA) together with 5'-primer A (CAAATTT AACGGGTCCA CGCAAGAA), which were designed from the upstream genomic sequence of maize B73's *MIPS-1s* gene. The primers were designed using the software Primer 5.

In silico promoter analysis

The promoter sequence of the B73 line was downloaded from the MaizeGDB database (B73 RefGen_v2) from positions -2,000 bp to +1. Promoter sequences for the other three inbred lines (Qi319, Chang7-2 and Chang3154) were obtained by PCR amplification and sequencing. The upstream regions were analyzed for the presence of putative cis-acting elements using PlantCare tools (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002). Sequence alignment was carried out using ClustalW2, and phylogenetic trees were constructed using MEGA 4.0 software.

Analysis of physicochemical properties of MIPS-1s protein

The physicochemical properties of MIPS-1s were computed using ExPASy's ProtParam tool (http://web.expasy.org/protparam/). Its subcellular location was analyzed by WoLF PSORT (http://wolfpsort.org) Transmembrane analysis was predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and hydropathicity analysis was estimated using the ProtScale program (http://web.expasy.ch/tools/protscale). The secondary structure of MIPS-1s was predicted using the self-optimized prediction method with alignment (SOPMA) (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html).

Anti-MIPS antibody generation

The anti-MIPS-1s polyclonal antibody was generated by immunizing healthy rabbits, using synthesized peptides (TPVVNALAKQRAMLENIMR) as antigens. The protein conjugations, immunizations, and antiserum purifications were carried out by BPI (Beijing Protein Innovation Co., Ltd, Beijing, China).

Isolation of total proteins

Maize leaves and seeds were ground manually to fine powder in liquid nitrogen. An 800 μL aliquot of extraction buffer (62.5 mM TRIS-HCl (pH 7.4), 10% glycerol, 0.1% SDS, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5% (v/v) b-mercaptoethanol) was added to each 300 mg sample of powder. The mixture was vortexed and then chilled on ice for 30 min. Samples were centrifuged at 12 000 rpm for 30 min at 4 °C, and the supernatants were transferred into fresh tubes and stored at –20°C for use in SDS-PAGE and WB analysis.

Western blot (WB) analysis

Equal amounts of maize protein from different tissues/organs were separated using 10% SDS-PAGE, and transferred onto a polyvinylidene difluoride (PVDF) membrane using an electrophoretic transfer system (Bio-Rad, USA) at 160 V for 65 min. WB analysis was performed according to established methods (Bai et al. 2012). Equal loading of proteins was confirmed by detection of HSP (Li et al. 2011). WB and quantification analyses were performed at least three times. The X-ray films were scanned and the intensity of each band was captured using an ImageMaster 2D Platinum version 5.0 (GE Healthcare Amersham Bioscience, Piscataway, NJ, USA). The intensity of each band was standardized as a percentage of the total intensity, and the results were referred to as a relative volume that represents the relative expression abundance of the gene in the samples tested. The relative expression abundance was calculated and plotted for each protein band, and significant differences in expression levels among different proteins were analyzed with SPSS software (SPSS, Quarry Bay, Hong Kong).

Results and discussion

Characterizing the genomic sequence of Qi319 MIPS-1s

Maize is an important crop and a model plant for biological research. Due to its economic significance, great effort has been taken in sequencing the genome. With the availability of the maize genome sequence, it is much easier to obtain coding or promoter sequences for a specific gene in maize lines, without having to conduct sequencing. Mutants that have

impaired accumulation of phytic acid have been reported in maize, and they show reduced germination rates and lower stress tolerance in seeds (Shi et al. 2007). We previously isolated a recessive maize mutant, Qi319, that had a dramatic reduction in grain phytic acid (65% lower than Chang 7-2). The germination rate of Qi319 seeds was > 75%, lower than the rate for most control lines of inbred maize, however, no other negative effects were evident in plant development and seed maturation (Gao et al. 2013).

Evaluation of the sequence features of the *MIPS-1s* gene isolated from Qi319, following PCR amplification (Fig. 1A), showed that the gene is 3588 bp in length, including 10 exons and 9 introns. While the *MIPS* genomic sequence of B73 contains 3618 bp. The cDNA sequence of the *MIPS-1s* gene encodes an open reading frame (ORF) of 1533 bp, a 95-bp 5' untranslated region (UTR), and a 47-bp 3'UTR. The detailed positions of the 10 exons were as follows: exon 1, 96-284; exon 2, 875-943; exon 3, 1038-1174; exon 4, 1600-1846; exon 5, 2209-2435; exon 6, 2520-2634; exon 7, 2731-2907; exon 8, 2992-3181; exon 9, 3270-3333 and exon 10, 3424-3541 (Fig. 2). In comparing the peptide sequence between MIPS-1s proteins in Qi319 versus B73 (GenBank: Accession AAG403281.1), there was a single amino acid substitution (valine to isoleucine) (Fig. 3), while the overall structure and sequence of MIPS-1s in the two

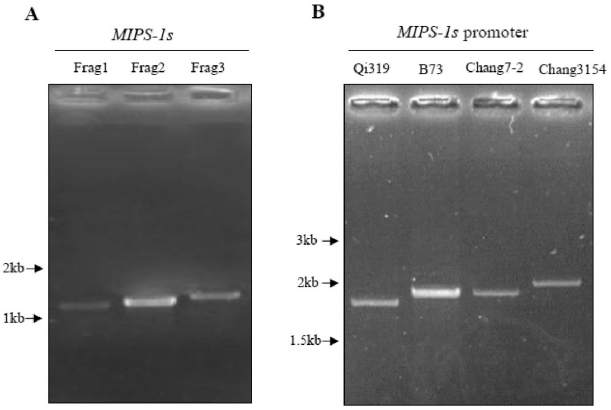

Fig. 1. PCR amplification of the *MIPS-1s* genomic sequence. A = PCR amplification of the genomic sequence from Qi319. The entire *MIPS-1s* genomic sequence was amplified using three pairs of primers; lengths of the three fragments were 1155bp, 1205bp and 1298bp, respectively; B. PCR amplification of the promoter region of *MIPS=1s* in four inbred lines of maize (Qi319, B73, Chang 7-2 and Chang 3145)

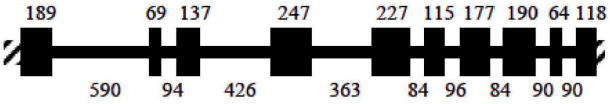

Fig. 2. Schematic diagram of the *MIPS-1s* gene in Qi319 maize. Exons are represented by black boxes and introns are highlighted by the solid line. The 5′ and 3′ UTR are shown in hatched bars. Exon lengths are given above the boxes in base pairs, and intron lengths are given below the solid lines

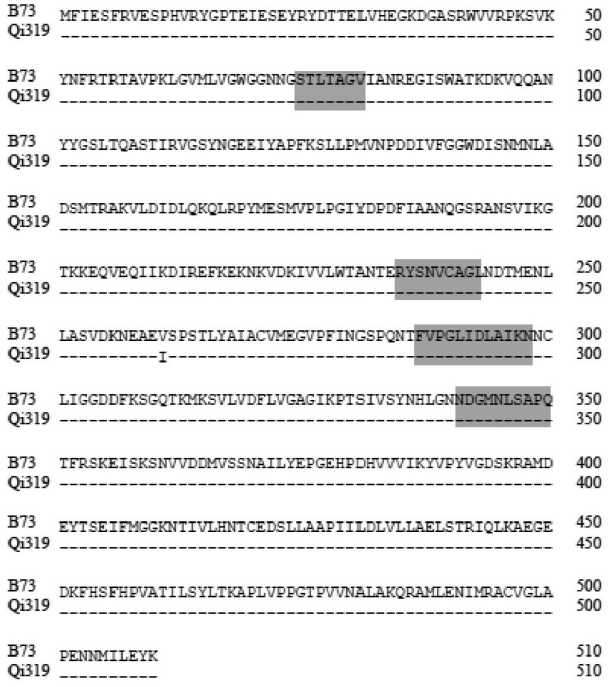

Fig. 3. Alignment of MIPS-1s amino acid sequences of Qi319 and B73. Four conserved MIPS functional domains are highlighted in gray

inbred lines was similar. Based on the sequence information, it is unlikely that the substitution from valine to isoleucine changed the enzyme activity dramatically, as the biochemical and physical properties of the two residues were quite similar. In summary, we reported the isolation and molecular characterization of the *MIPS-1s* gene involved in phytic acid synthesis in Qi319 maize. Its overall structure and sequence was quite similar with previously reported maize *MIPS* (AAG40328.1), with one amino acid substitution.

Physicochemical properties of the MIPS-1s protein

To better understand its physicochemical properties, the MIPS-1s protein was analyzed with a number of

tools. The deduced pre-mature peptide of MIPS-1s consisted of 510 amino acid residues, with a predicted molecular weight of 56.3 kDa and an estimated isoelectric point (pI) of 5.65. Its instability coefficient was 33.71, suggest that MIPS-1s is a stable protein. The total average hydropathicity was –0.184. ProtScale analysis showed that the hydrophilic amino acid is distributed over the peptide symmetrically (Fig. S1). MIPS-1s was predicted to be a cytoplasmic subcellular localized protein by WoLF PSORT, while no transmembrane domain was found in MIPS-1s (data not shown). SOPMA analysis revealed that the overall folding of the protein is basically built with á-helices (37.65%) and random coils (37.65%) that are connected with extended strands (18.82 %) and β-turns (5.88 %) (data not shown). Four highly conserved domains (GWGGNNG, LWTANTERY, NGSPQNTFVPGL and SYNHLGNNDG) were found and highlighted in MIPS-1s (Fig. 3). Domain I (GWGGNNG) is reportedly responsible for binding of co-factor NAD^+ while domain IV (SYNHLGNNDG) binds with its substrate (Ma et al. 2013).

In silico promoter analysis

Sequencing of the *MIPS-1*s promoter and investigation of its regulatory features, following PCR amplification of the promoter region in the four maize lines (Fig. 1B), showed that the amplified upstream promoter region had a length of 1726 bp, 2011 bp, 1945 bp and 2125 bp in Qi319, B73, Chang7-2 and Chang3154, respectively. When sequences were aligned by ClustalW2 and the phylogenetic trees were constructed, the sequence of the promoter region of Qi319 was quite different from those of B73, Chang7-2 and Chang3154, while the sequence of the promoter regions of B73 and Chang7-2 were highly similar (Fig. 4). The PlantCARE analysis showed that all four sequences had the typical characteristics of gene promoters, containing 41, 40, 40 and 39 regulatory elements respectively (Table 1). The predicted regulatory elements can be categorized by function

Fig. 4. Phylogenetic tree of the MIPS-1s promoter region from four inbred maize lines (B73, Chang 7-2, Qi319 and Chang 3154) generated by MEGA 4.0 software

Table 1. Cis-acting elements in putative promoter sequences of the *MIPS-1s* gene in four lines of inbred maize

cis element	Qi319	B73	Chang 7-2	Chang 3154	Function
3-AF1 binding	+	+	+	+	Light-site responsive
AAGAA-motif	+	+	+	+	
ACE	+	–	–	–	
AE-box	+	+	+	–	
Box 4	+	+	+	+	
Box I	+	+	+	+	
G-box	+	+	+	+	
G-BOX	+				
I-box	+	+	+	+	
LAMP-element	+				
CATT-motif	+	+	+	+	
GAG-motif	+	+	+	+	
GATA-motif	+	+	+	+	
GT1-motif	+	+	+	+	
MNF1	+	+	+	+	
Sp1	+	+	+	+	
ATCT-motif	–	+	+	–	
MRE	–	+	+	+	
circadian	+	+	+	+	
ABRE	+	+	+	+	ABA-responsive
TGACG-motif	+	+	+	+	MeJA-responsive
CGTCA-motif	+	+	+	+	
GARE-motif	+	+	+	+	Gibberellin-responsive
TATC-box	+	–	–	+	
TCA-element	+	+	+	+	Salicylic acid-responsive
TC-rich repeats	+	+	+	+	Defense and stress
ARE		+	+	+	Anaerobic induction
HSE	+	+	+	+	Heat
LTR	+	+	+	+	Low temperature
MBS	+	+	+	–	MYB
GC-motif	+	+	+	+	Anoxic
TATA-box	+	+	+	+	Core element
CAAT-box	+	+	+	+	
5UTR Py-rich stretch	–	+	+	+	High transcription levels

MSA-like	–	+	+	+	Cell cycle
Box III	+	+	+	+	Protein binding site
Skn-1_motif	–	–	–	+	Endosperm
O2-site	–	–	–	+	Zein
MBSII	+	+	+	+	Flavonoid
CCGTCC-box	+	–	–	–	Meristem
Unnamed__1	+	+	+	+	Unknown
Unnamed__2	+	+	+	+	
Unnamed__3	+	+	+	+	
Unnamed__4	+	+	+	+	
TCCACCT-motif	+	+	+	+	
box S	+	+	+	+	
box E	–	+	+	–	
OBP-1 site	+	–	–	–	
A-box	+	–	–	–	
Total number	41	40	40	39	

"+" and "–" indicate presence and absence of the *cis*-element; *cis*-elements unique to the Qi319 promoter region are bolded.

into tissue-specific, biological stress responsive, abiotic stress responsive, stress hormone responsive, growth hormone responsive and basic regulatory elements. Light responsive elements, ABA, MeJA, gibberellin and salicylic acid growth hormone responsive elements, heat shock, low temperature and anoxia responsive elements were also observed. Notably, a number of cis-elements were located specifically in the promoter region of Qi319, including ACE, G-Box, LAMP-element, CCGTCC-box, OBP-1 site and A-box, while a number of other cis-elements were absent only in the promoter region of Qi319. Taken together, these data support the idea that the promoter region of *MIPS-1s* may be responsible for the reduction of phytic acid in Qi319. However, based on current information, we cannot address whether the haplotype of the sequence may be responsible for the difference in expression. Detailed pinpoint analysis will be needed to determine which cis-element specifically controls the expression of MIPS-1s, leading to the low phytic acid phenotype.

Gene regulation is mediated by the presence of cis-acting (promoters), or trans-acting (DNA-binding protein) elements, yet to date there has been limited molecular information about the maize *MIPS-1s* gene promoter. Shukla et al. (2004) speculated that a mutation in the *MIPS* promoter might diminish or prevent binding of RNA polymerase. In the present study, we isolated and sequenced the *MIPS-1s* promoter regions. PlantCARE predicted elements of known function that interact with transcription factors to enhance or repress gene expression. Light responsive elements were reported among the most frequently observed ones suggesting that transcriptional regulation might lead to diurnal fluctuations of *MIPS* transcript levels and enzyme activity in iceplant, potato and rice (Ishitani et al. 1996; Ray Chaudhuri and Majumder 1996; Keller et al. 1998). ARE, HSE, LTR and MBS elements involved in abiotic stresses-specific expression are also present in the *MIPS-1s* promoter sequence, indicating that MIPS may play roles in plant growth, development, and defense against stress. MIPS was recently found to increase in leaves after salinity stress (Nelson et al. 1998), and similarly, transcript levels of the *MIPS* gene increased immediately after 10 min of heat stress (Khurana et al. 2012). The MIPS enzyme is necessary for myo-inositol production, and it is the first and rate-limiting substance in the synthesis of phytic acid. In rice, Yoshida et al. (1999) detected MIPS transcript in the early stage of seed development. MIPS transcript (*RINO1*) was observed in developing rice seeds from 2 DAA (days after anthesis) to 10 DAA. In soybean, GmMIPS-1 was expressed in maternal tissues and then in the developing embryo (Hegeman et al. 2001). In a recent report (Ma et al. 2013), MIPS activity was closely related to the expression of the *MIPS* gene. This might explain the increase in phytate concentration observed during seed development. In this study, a number of cis-elements were found specifically at the promoter region of MIPS-1s of Qi319, providing evidence that the region may play a key role in the regulation of differential expression of MIPS-1s protein that was revealed by WB analysis.

Expression analysis of MIPS-1s protein at different developmental stages

Western blot was used to investigate the expression pattern of MIPS-1s protein at different developmental stages (Fig. 5A); a single band, with a predicted molecular weight of 56.3 kDa, was detected in leaves, pollen and immature seeds of Qi319, which demonstrated the specificity of the antibodies. A similar expression pattern of MIPS-1s proteins as obtained at different developmental stages in Chang7-2 (Fig. 5B). The highest level of expression occurred in pollen and seeds, while expression of MIPS-1s was weak in older leaves at the anthesis stage, and no signals were detected in young leaves nor in young and older roots. Interestingly, the size of band did not match the predicted molecular weight in immature

tassels, in both inbred lines, suggesting that alternative MIPS-1s isoforms existed in the tissue. The fact that MIPS-1s protein was expressed under normal growth conditions in different tissues supports the notion that it plays similar roles in maize development and growth, in both low and normal phytic acid inbred lines of maize.

Phytic acid is known to accumulate in seeds, and to further understand the relationship between MIPS-1s protein expression and phytate synthesis, WB analyses were conducted to compare the expression of MIPS-1s in immature seeds of *lpa* mutant and normal inbred controls. Total proteins were separated by SDS-PAGE and then analyzed by WB using anti-MIPS antibodies (Fig. 6); the MIPS-1s signals were detected in 10 DAF and the peak signals appeared in 15 DAF, followed by a slight decrease at 25 and 30 DAF (Fig. 6A). Similar results were obtained in Chang7-2 kernels (Fig. 6B), in which the initial expression was detected in 3 DAF and the signal increased in the following stages; peak

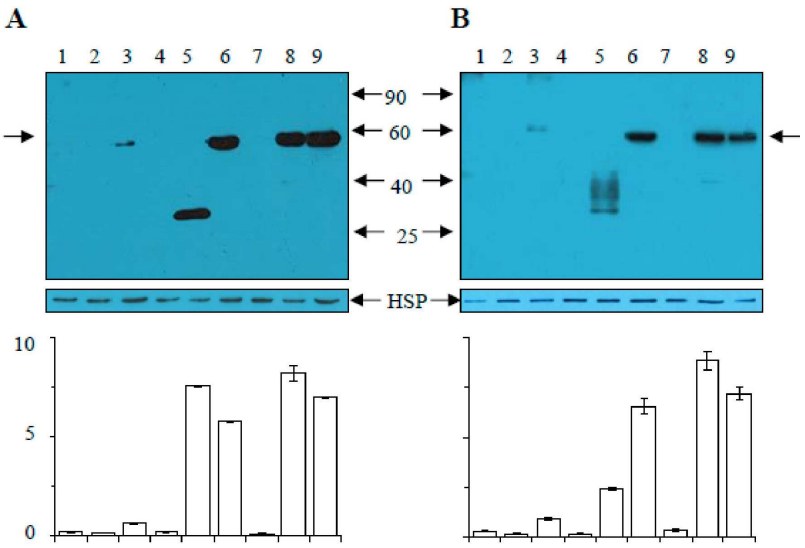

Fig. 5. Expression profiling of MIPS-1S proteins in different tissues of Qi319 (A) and Chang7-2 (B) maize lines. Lane 1: leaves at seedling stage; Lane 2: roots at seedling stage; Lane 3: leaves at anthesis stage, Lane 4: roots at anthesis stage; Lane 5: tassel; Lane 6: pollen; Lane 7: pistil; Lane 8 and 9: 15 and 30 days after fertilization, respectively. Upper panels: Western Blot (WB) detection of the expression of MIPS-1S protein. Middle panels: WB results detected by anti-HSP antibody to demonstrate equal loading. Lower panels: Plot of average and standard deviation among three repeats of WB analysis for MIPS-1S protein (the band highlighted by arrows)

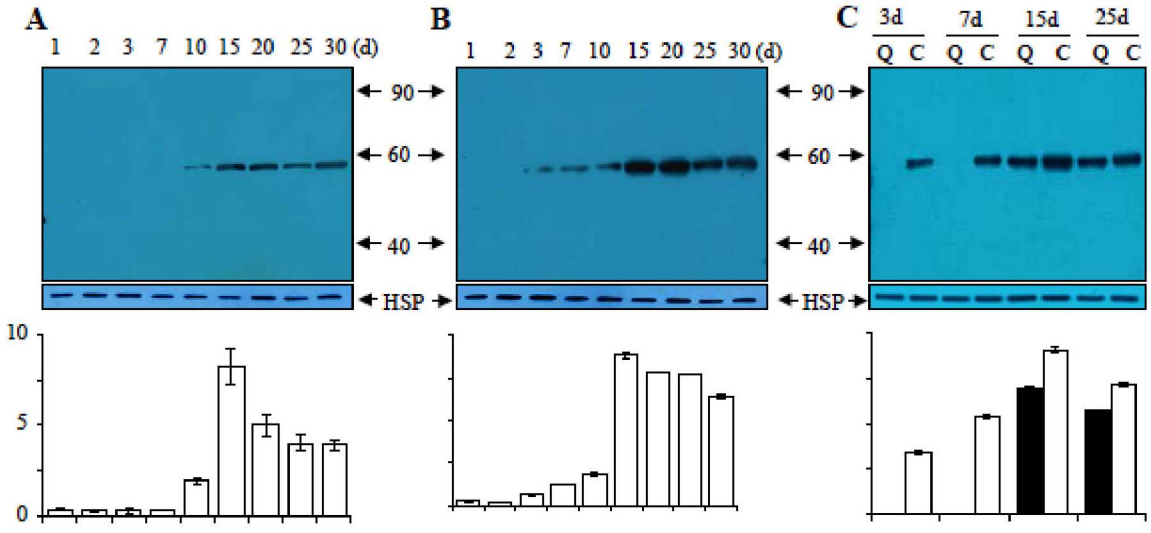

Fig. 6. Expression profiling of MIPS-1S proteins in developing maize seeds. A = Qi319; B = Chang7-2; C = Side by side comparison of MIPS-1S protein abundance between Qi319 (Q) and Chang 7-2 (C). Numbers above the figures indicate days after fertilization. Upper panels = Western Blot (WB) detection of the expression of MRPS-1S protein. Middle panels = Signals generated using anti-HSP antibody was used as an equal loading control. Lower panels = Plot of average and standard deviation among three repeats of WB analysis

expression appeared at 15 DAF, followed by a slight decrease at 25 and 30 DAF.

To quantitatively compare the MIPS-1s protein abundance between Qi319 and Chang7-2, side by side WB were performed in the same gel for four representative time points (Fig. 6C). The results clearly showed a stronger expression in Chang7-2, especially during the early period. The data showed that inbred maize with higher phytic acid content had higher levels of MIPS-1s protein expression as compared to inbred maize with lower phytic acid content. This finding suggested that higher levels of MIPS expression should precede and/or accompany the synthesis and accumulation of phytate.

Using immunological techniques, MIPS-1s expression was detected in pollen, leaves and seeds, but the strongest differences were observed in pollen and immature seeds. Abreu and Aragao (2007) reported the presence of *PeMIPS1* transcripts in pollen, leaves and immature seeds using RT-PCR. In soybean, Hegeman et al. (2001) reported higher expression of MIPS in immature seeds compared to other tissues, based on northern blot analysis. Yoshida et al. (1999) showed rice *MIPS* transcripts were detected 4 days after anthesis, with peak signals at 7 DAA, after which the signals decreased gradually. Ma et al. (2013) detected the *MIPS* transcripts signal in immature seeds at 14 DAF. In that study, immunoblot analyses indicated that maize with higher phytic acid content had higher levels of MIPS protein expression, but the maize with lower phytic acid content had lower levels during seed development. Pilu et al. (2003) used RT-PCR to compare MIPS expression between wild-type and mutant seedlings, and a lower level of expression was observed in the mutant. The mutant soybean lines LR33 with reduced MIPS activity experienced a block in seed phytic acid synthesis (Hitz et al. 2002). Overall, these data suggest that the *MIPS-1s* gene plays a key role in supplying inositol for early seed development and phytic acid accumulation.

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