African Journal of Biotechnology Vol. 11(98), pp. 16443-16454, 6 December, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2811
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Spatial and temporal expression analysis of D-*myo*-inositol 3-phosphate synthase (*MIPS*) gene family in *Glycine max*

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Accepted 29 November, 2012

Phytic acid, the principal storage form of phosphorus in plant seeds accounts for up to 60 to 80% of the total seed phosphorus content in soybean. Its accumulation increases linearly throughout seed development and it strongly chelates essential mineral cations and charged proteins decreasing their bioavailability. D-*Myo*-inositol 3-phosphate synthase (MIPS; EC 5.5.1.4), the evolutionarily conserved enzyme in plants, catalyzes the first and the rate limiting step in phytic acid biosynthetic pathway. Aiming at controlling the level of phytate, we monitored the differential expression profile of four, previously reported, members of the *MIPS* gene family in developing seeds and vegetative tissues of soybean by quantitative real-time PCR (qRT-PCR). Transcript levels were measured relative to the endogenous reference gene eEF-1α (eukaryotic elongation factor 1-alpha) using SYBER-Green. The qRT-PCR data analysis indicated that the expression of the four highly conserved *MIPS* genes is both temporally and spatially regulated, information much needed for reverse genetic applications. *MIPS1* exhibited high transcript levels in the early developing cotyledons with the levels peaking at around 4 to 6 mm seed size stage. Despite of high level of nucleotide sequence conservation amongst the *MIPS* gene family members, *MIPS2, MIPS3* and *MIPS4* were poorly expressed in developing seed tissues, although their transcript levels were relatively high in the other organ tissues. *MIPS1* was however moderately expressed in seedlings where *MIPS2* showed relatively higher expression levels. Among the four isoforms, *MIPS4* had the highest transcript levels in the leaf tissue. The data was clearly indicative of the fact that the four isoforms had diverged regulatory elements resulting in their differential expression. Of the four members of the *MIPS* gene family, *MIPS1* is thus the major isoform that had high expression in the developing seed tissues and can be targeted using the dsRNA induced sequence specific RNA degradation mechanism for reduction of phytate levels without affecting the critical aspects of inositol metabolism in other tissues of the plant.

Key words: Soybean, *MIPS* isoforms, differential expression, endogenous reference gene, qRT-PCR.

INTRODUCTION

Phytic acid (*myo*-inositol hexa*kis*phosphate, InsP6) is the major storage form of phosphorus in plant seeds. It begins to accumulate in seeds after the cellular phosphate levels have reached their maximum levels and continues to increase linearly throughout seed development and seed filling (Raboy and Dickenson, 1987). Phytic acid is usually deposited in protein bodies as a mixed salt (phytate), bound to mineral cations such as Fe^{3+}, Ca^{2+}, Mg^{2+}, Zn^{2+} and K^{+} (Prattley and Stanley, 1982; Lott, 1984). In soybean seeds, 60 to 80% of total phosphorus is present as phytic acid which can account for up to 2% of the soybean seed dry weight (Raboy et al., 1984). During seed germination, plants mobilize

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phosphate, inositol and mineral cations from phytic acid for utilization in growth and development by specific expression of the phytic acid degrading enzyme phytase (Strother, 1980; Raboy, 1997).

Although phytic acid is an important storage molecule for growing seedlings, it poses several problems for agriculture. It acts as an antinutrient in the digestive tract of both ruminants and nonruminants, chelating mineral cations, including calcium, zinc, magnesium and iron thus affecting the bioavailability of these essential minerals (Raboy, 1997). Phytic acid also has the potential to bind charged amino acid residues of proteins, with the concomitant reduction of protein availability and digestibility (Raboy, 1990).

The nutritional problem is especially serious with legume crops such as soybean, because the phytic acid remains tightly bound to reserve proteins during standard processing procedures. These antinutritional qualities of phytate can be further extended to human health as they contribute to the iron deficiency suffered by over 2 billion people worldwide (Bouis, 2000).

Monogastric livestocks lack sufficient phytase activity to metabolize the phytate present in cereal seeds, leading to inadequate phosphorous uptake and the need to supplement animal feed with expensive and non-renewable inorganic phosphorus (Brinch-Pedersen et al., 2002).

Phytic acid and complexed nutrients pass through the digestive tract of nonruminants and are excreted in their manure. Repeated applications of phytic acid containing animal waste as fertilizer runoff from fertilized fields transfers high concentrations of phosphorus to groundwater, leading to eutrophication of lakes, ponds and streams (Raboy, 2007). The undesirable properties of phytic acid listed above make the development and characterization of low phytate crops a high priority in agricultural research.

Reduction of phytic acid levels in soybean seeds would not only obviate the need to supplement livestock feed with phosphorus but also improve the nutritional quality of soybean meal. Lower phytic acid levels in seeds used for soybean meal would also decrease phytic acid levels in animal waste, reducing overall phosphorus pollution in areas of intensive livestock production. The most common strategy to improve phosphorus bioavailability is the use of supplemental microbial phytases.

Unfortunately, microbial phytase supplementation of livestock diets is even more costly than direct addition of inorganic phosphorus to meet nutritional requirements. Costlier alternatives to reducing phytic acid levels, such as direct addition of fungal phytase supplements to feed, could be eliminated with the introduction of low phytic acid feeds. Low phytic acid (lpa) mutants have been identified in several important crop species and one such soybean mutant, LR33, exhibited a 50% reduction in seed phytic acid levels (Hitz et al., 2002). However, conventional breeding strategies using mutants result in poor agronomic properties, such as decreased germination and reduced seedling emergence (Raboy, 2007). Therefore, a different approach is required to overcome these potential problems.

An alternative strategy for decreased seed phytate levels is the reduction of the expression of enzymes in the biosynthetic pathway of phytic acid. D-*Myo*-inositol-3-phosphate synthase (MIPS EC 5.5.1.4) catalyzes the first committed and the rate-limiting step (Coelho and Benedito, 2008; Chen and Xiong, 2010) in *de novo* synthesis of *myo*-inositol and phytate biosynthesis that is NADH-dependent conversion of glucose-6-P to myo-inositol-3-P (Loewus and Murthy, 2000).

MIPS is a highly conserved enzyme that has been identified throughout all biological kingdoms in such diverse organisms as Archaebacteria, eubacteria, yeast, plants, flies and humans (Majumder et al., 1997, 2003; Bachhawat and Mande, 2000). The characterization and localization of MIPS enzymes has been conducted by several research groups (Abreu and Aragao, 2007; Chiera and Grabau, 2007; Mitsuhashi et al., 2008). *MIPS* structural genes have been isolated, cloned and sequenced from a variety of microbial plant and animal sources, including yeast (Johnson and Henry, 1989), potato (Keller et al., 1998), *Spirodela polyrrhiza* (Smart and Fleming, 1993), *Oryza sativa* (Yoshida et al., 1999; Hait et al., 2002), *Arabidopsis* (Johnson, 1994), *Vigna radiata* (Adhikari et al., 1987; Wongkaew et al., 2010), *Passiflora edulis* (Abreu and Aragao, 2007) and *Glycine max* (Iqbal et al., 2002; Kumar et al., 2012).

Although yeast and animal genomes contain a single *MIPS* gene (Donahue et al., 2010), multiple genes for *MIPS* have been reported in plants (Torabinejad and Gillapsy, 2006; Donahue et al., 2010). In soybean four isoforms of *MIPS* gene with their complete genomic sequences and their expression profiles have been examined by qRT-PCR (Chappell et al., 2006). In maize, seven sequences homologous to *MIPS* have been mapped to different chromosomes (Larson and Raboy, 1999).

Three *MIPS* genes identified in *Arabidopsis* are expressed in siliques, leaves and roots (Mitsuhashi et al., 2008). The multiple *MIPS gene* isoforms in plant species may be applied to attune their differential expression to specific physiological functions thereby making it essential to know which genes in the *MIPS* family are expressed in seeds for targeting them by gene silencing strategies to obtain low phytate phenotypes.

For achieving this ultimate goal, the present study was carried out to monitor spatial and temporal variation in the expression pattern of four members of the *MIPS* gene family, in both the developing seeds and other organ tissues of soybean, by quantitative real-time PCR (qRT-PCR) for developing RNAi and antisense strategies with the targeted isoforms to obtain low phytate soybean seeds without affecting major critical aspects of inositol metabolism in other tissues of the plant.

Table 1. Primers used for qRT-PCR..

Target	Name	Sequence (5'→3')	Direction
MIPS1	MIPSl-exp-F	CATCGAGAATTTTAAGGTTGAGTGTCC	Forward
	MIPSl-exp-R	CCTAATTTAGGAACATGGATGTTGGTT	Reverse
MIPS2	MIPS2-exp-F	CATCGAGAATTTTAAGGTAGAGAGTCC	Forward
	MIPSl-exp-R	CCCAATTTTGGAACATGGGTGTTGGTT	Reverse
MIPS3	MIPS3-exp-F	CATCGAGAGTTTCAAGGTTGAGAGTCC	Forward
	MIPS3-exp-R	CCTAGTTTAGGGACATGGGTGTTGGTT	Reverse
MIPS4	MIPS4-exp-F	TATGTACCTTACGTTGGGGACAGCAAG	Forward
	MIPS4-exp-R	CTCATTTTCAGCTTTAAACTCGATTCG	Reverse
eEF-1α	EF1F	TGTTGCTGTTAAGGATTTGAAGCG	Forward
	EF1R	AACAGTTTGACGCATGTCCCTAAC	Reverse

MATERIALS AND METHODS

Plant materials

Developing pods of soybean (*G. max* L. *var*. Pusa16) were collected from the fields of Division of Genetics, Indian Agricultural Research Institute, New Delhi, India. The variety Pusa-16 with an area of adaptability in the Northern plain and Northern hill zones has a maturity duration of 105-115 days. For expression analysis, various tissues of the soybean plants including seedling, flowers, roots, stems, leaves and developing cotyledons were harvested from the fields and rapidly frozen in liquid nitrogen before storing them at -80°C.

RNA isolation and quantification

For quantitative expression analysis, the developing seeds were divided into groups based on length as measured from apical end to basal end of the seed (0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 10 and 10 to 12 mm). Frozen plant tissues were homogenized using pestle/mortar in liquid nitrogen and 1 ml of Qiagen lysis buffer was added per 100 mg of tissue in 2 ml micocentrifuge tubes. Total RNA was isolated from the developing cotyledons and tissue samples (seedlings, root, stem, and leaves) of soybean samples (100 mg) using RNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. An aliquot of each RNA sample was analysed on 1% agarose gel for confirming the intactness of the ribosomal RNA bands. RNA sample concentrations were measured twice on NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and the samples were adjusted to the same concentration between each measurement. The samples were stored at -80°C. Similar volumes of accurately quantified RNA were thus entered in each reverse transcription reaction, thereby homogenizing RNA input quantity in the subsequent cDNA synthesis reaction during quantitative real-time PCR (qRT-PCR) analysis.

Differential gene expression by qRT-PCR

Expression levels of four isoforms in soybean *MIPS* gene family were normalized to housekeeping gene eEF-1α (eukaryotic elongation factor eEF-1α; GenBank accession No.X56856) which was used as an internal control gene. Exonic sequences of previously reported complete genomic sequences of *MIPS1*, *MIPS2*, *MIPS3* and *MIPS4*, deposited in GenBank (accession numbers DQ323904, DQ323906, DQ323907 and DQ323908) were used for primers designing and the primers were ordered from Sigma-Aldrich. Primer sequences of the four isoforms are shown in Table 1. The qRT-PCR was conducted according to standard protocol using the SuperScript III Platinum SYBER Green One-Step qRT-PCR Kit (Invitrogen). For each qRT-PCR reaction, 25 µl mixture containing 2 µl diluted RNA (50 ng RNA), 12.5 µl of SYBER Green PCR master mix and 20 ρM of each of the forward and reverse primers were used. Thermal cycling conditions comprised 50°C for 1 h followed by an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. All qRT-PCR reactions were preformed in the Stratagene Mx3000p followed by analysis of the dissociation curve, taking a florescence reading at every degree between 55 and 95°C, to ensure the amplification of only one amplicon. Gene of interest and internal control gene were measured in separate wells with accurate pipetting to avoid erroneous normalization by the reference gene and all experiments were performed in triplicate. The data analysis was done using Mxpro-QPCR software (Stratagene).

In silico sequence analysis

The amino acid sequence of the four members of the *G. max MIPS* gene family were subjected to basic local alignment search tool (BLAST) analysis using ClustalW and BOXSHADE softwares for multiple alignment of these isoforms with the other plant *MIPS*.. The phylogenetic analysis of nucleotide and protein sequences was carried out using neighbor-joining (N-J) method with 1000 bootstrap replicates implemented in MEGA (Molecular Evolutionary Genetic Analysis) version 5 software program (Tamura et al., 2011).

RESULTS AND DISCUSSION

Characterization of the *Glycine max MIPS* gene family

Previous studies on DNA blot analysis had revealed multiple copies of the *MIPS* gene within the soybean genome (Hegeman et al., 2001). Four *MIPS* expressed

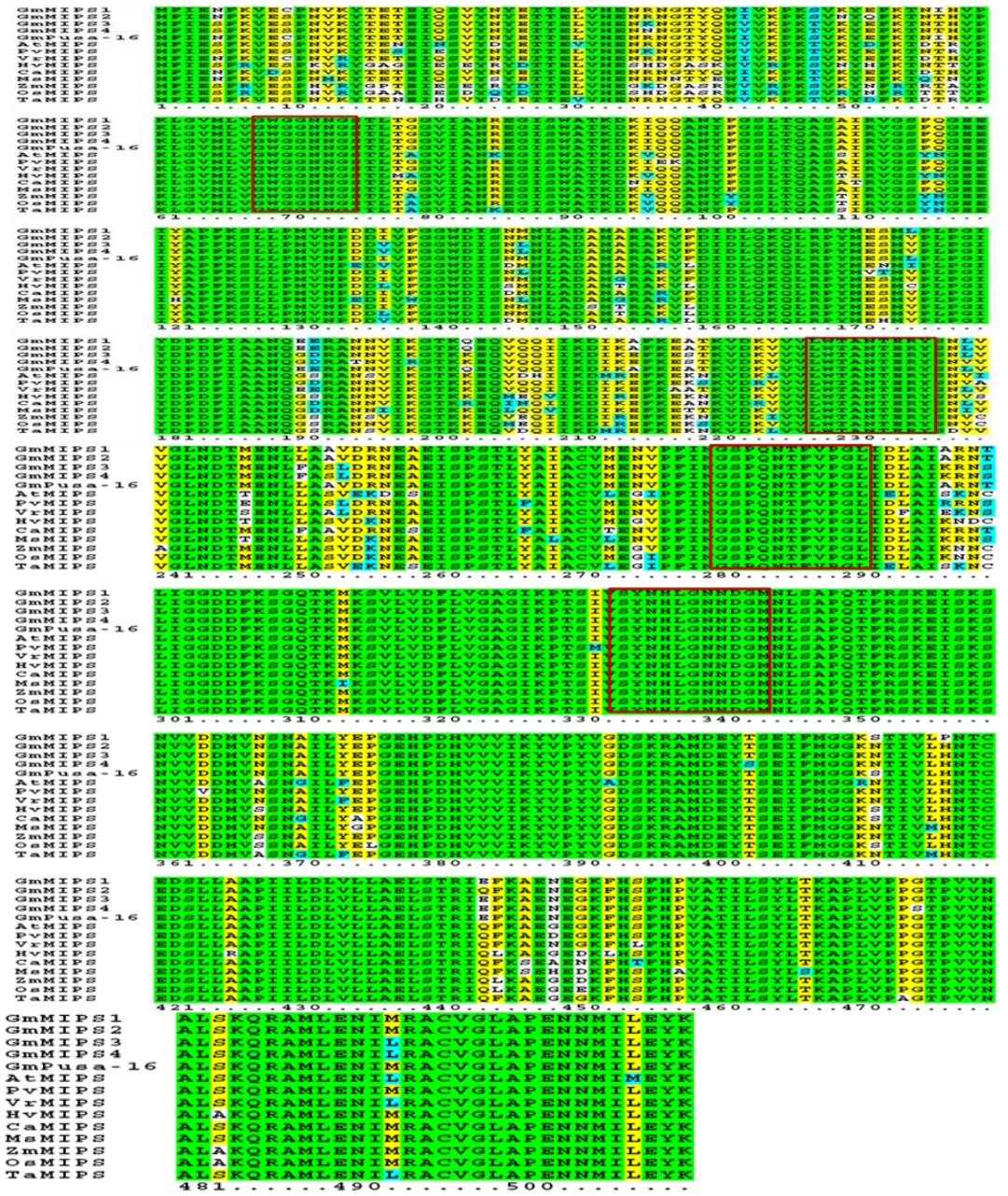

Figure 1. Alignment of deduced amino acid sequences of plant MIPS showing four highly conserved domains (shown in boxes). GenBank database accession numbers of the displayed *MIPS* genes are as follows: *G. max* gene family isoforms (GmMIPS1: ABM17058, GmMIPS2: ABC55420, GmMIPS3: ABC55421, GmMIPS4: ABC55422, GmPusa-16: ADJ38521), *A. thaliana* (AtMIPS: AAD23618), *P. vulgaris* (PvMIPS: ACN12926), *V. radiata* (VrMIPS: ABW99093), *H. vulgare* (HvMIPS: O65195), *C. arietinum* (CaMIPS: ACH87552), *M. sativa* (MsMIPS: ABO77439), *Z. mays* (ZmMIPS: ACG33827), *O. sativa* (OsMIPS: NP_001049242), *T. aestivum* (TaMIPS: AAD26330).

sequence tags (ESTs) (*GmMIPS*) with differential tissue specific expressions were reported in soybean (Hegeman et al., 2001). The complete genomic sequences of the four isoforms, *MIPS1, MIPS2, MIPS3* and *MIPS4* were deposited in GenBank (accession numbers DQ323904, DQ323906, DQ323907 and DQ323908) by Chappell et al. (2006). In the present study, protein sequence analysis using BOXSHADE software for multiple alignment of the *MIPS* isoforms of soybean demonstrated the presence of four highly conserved motifs: GWGGNNG / LWTANTERY / NGSPQNTFVPG and SYNHLGNNDG confirming a high degree of conservation among the members of *MIPS* gene family (Figure 1). The amino acid sequence of these four domains were also

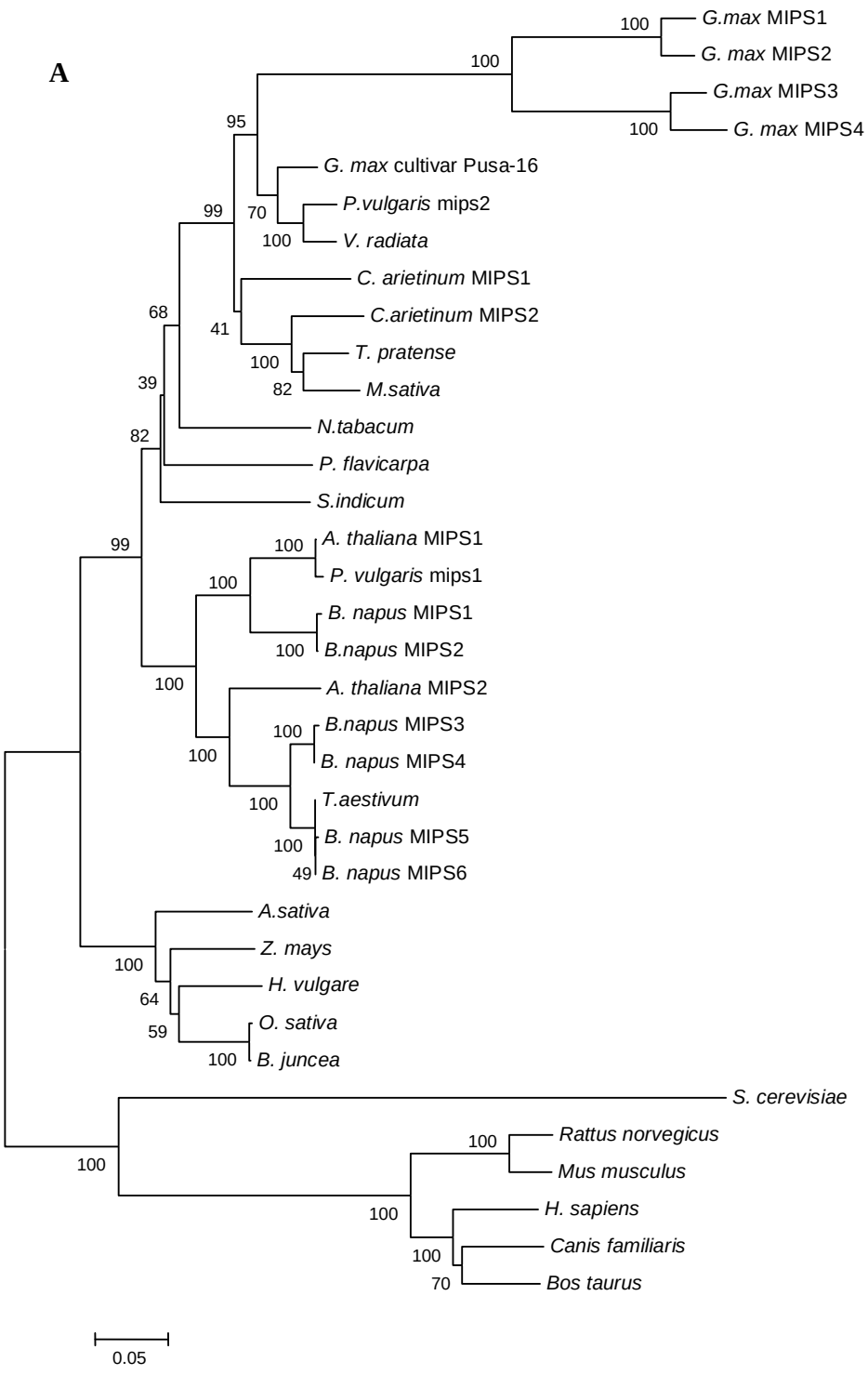

Figure 2. A) Phylogenetic tree of eukaryotic *MIPS* based on nucleotide sequence. B) Phylogenetic tree showing relationships among eukaryotic MIPS proteins.

found to be identical to those of other plant MIPS. Multiple alignment of the four isoforms of GmMIPS protein sequences showed 99% identity of MIPS2 with MIPS1 whereas MIPS3 and MIPS4 were 96% and 95% identical to MIPS1 respectively. The highest scoring eukaryotic *MIPS* nucleotide and protein sequences from BLAST search were used to generate a phylogenetic tree (Figures 2a and b) using the neighbour-joining (N-J)

method with 1000 bootstrap replicates implemented in the MEGA 5 programme (Tamura et al., 2011). The phylogenetic tree presented an overall evolutionary divergence of this gene in the biological kingdom with higher plants and higher animals forming two separate subgroups in the eukaryotic cluster. The analysis revealed high degree of homology among the members of soybean *MIPS* genes family and the other plant species at both protein as well as nucleotide sequence levels. On the phylogenetic tree, the *MIPS* genes sequences from plant species showed two main sub-branches of monocots and dicots with *Triticum aestivum* rooted surprisingly with dicotyledonous species. The legumes such as *G. max, P. vulgaris* and *V. radiata* were clustered on a single sub-branch of the tree while the other sub-branches were formed by species in the family Brassicaceae (*Arabidopsis thaliana, Brassica napus*) and Gramineae (*Zea mays*, *Triticum aestivum*, *Hordeum vulgare* and *O. sativa*). A high level of sequence conservation throughout the plant kingdom and also among all eukaryotic *MIPS* indicated *MIPS* evolution from a common stock. Sequence identity matrix showed that the deduced amino acid sequence of *GmMIPS1* cDNA had 96% identity with MIPS from *Vigna radiata*, 95% with *Phaseolus vulgare*, 94% with *Cicer arietinum*, 93% with *Nicotiana tabacum*, 88% with MIPS of *Z. mays*, 89% with *T. aestivum* and 87% with *A. thaliana.* Least sequence identity was however observed to yeast and *Homo sapiens* (6%) (Table 2). Multiple *MIPS*-coding genes have been reported in several plants which possibly allow differential expression during periods of high demand of inositol example seven *MIPS* sequences in maize (Larson and Raboy, 1999), two *MIPS* genes encoding the RINO1 and RINO2 in rice genome (Yoshida et al., 1999; Suzuki et al., 2007), three *MIPS* genes in *Arabidopsis* (Johnson and Sussex, 1995; Mitsuhashi et al., 2008), three copies in *Sesamum indicum* (Chun et al., 2003), four in soybean (Hegman et al., 2001; Chappell et al., 2006), two in chickpea (Kaur et al., 2008) and three isoforms of *MIPS* gene in *P. vulgaris* (Abid et al., 2012). Kaur et al. (2008) and Majee and Kaur (2011) in their comparative study on two divergent *MIPS* genes in chickpea revealed the features of both functional redundancy and diversification and hypothesized the multiplicity of *MIPS* gene to arise through gene duplication contributing to adaptive evolution. In the present study amino acid sequences encoded by *MIPS* gene isoforms in soybean demonstrated a high degree of conservation as the four highly conserved motifs common to both monocots and dicots plants were present in them. Earlier studies by Chun et al. (2003) and Abid et al. (2012) had also revealed that the amino acid sequences of these four domains were conserved in the MIPS proteins of all eukaryotes. The four domains, involved in MIPS protein binding were reported to be essential for MIPS functions, such as cofactor NAD^+ binding and reaction catalysis (Majumder et al., 1997; Norman et al., 2002). Based on the reported *MIPS* coding sequences from various organisms, Majumder et al. (2003) also proposed a highly conserved probable core catalytic structure conserved throughout the phylogenetic domain implying an essential function for the enzyme in cellular metabolism throughout the biological kingdom. Clubbed with the phylogenetic analysis, the present study indicated functional diversification of soybean *MIPS* which probably contributed to the adaptive evolution of the plant.

Differential expression profiles of *MIPS* gene isoforms in soybean

The existence of multiple *MIPS* genes in plant species may be responsible for assigning differential *MIPS* expression to specific physiological functions. However, only few studies have been reported on temporal and spatial expression profiles of *MIPS* gene family in various crops including rice (Yoshida et al., 1999), soybean (Chappell et al., 2006), chickpea (Kaur et al., 2008), *A. thaliana* (Mitsuhashi et al., 2008) and *P. vulgaris* (Lackey et al., 2003; Coelho et al., 2007; Abid et al., 2012). As a step towards elucidating the function of four *MIPS* genes within the soybean genome and to isolate and characterize the *MIPS* gene isoforms from the developing seeds, in the present study we examined their transcript accumulation patterns across different organ tissues through qRT-PCR. Total RNA isolated from various tissues was quantified for expression analysis (Figure 3) and the transcript levels of the four *MIPS* gene isoforms were normalized relative to the internal reference gene *eEF-1α* (eukaryotic elongation factor). All the four *MIPS* isoforms showed variation in both and spatial and temporal expression patterns in different organ tissues including developing seeds (Figure 4a). The isoform *MIPS1* showed highest transcript levels in the cotyledons at early stages of seed developmental with the expression showing a peak at around 4 to 6 mm seed size stage. As the development progressed, the expression decreased to nearly undetectable levels in the 10 to 12 mm seeds. In comparison the *MIPS1* transcripts were found in almost negligible amounts in other organ tissues that is roots, stems, leaves and flowers (Figure 4b). The expression analysis of *MIPS2* gene (Figure 4c) indicated higher levels of transcripts in the seedlings and root than in developing seeds. *MIPS3* and *MIPS4* expression was however (Figures 4d to e) the highest in leaves and flowers. Amongst the four isoforms, *MIPS4* showed the highest transcript levels in leaves. High levels of *MIPS1* mRNA in developing seeds and higher transcript levels of *MIPS3* and *MIPS4* in other tissues including stem, flowers, root and leaves demonstrated that the expression for the *MIPS* gene family members is organ specific and such diverged regulation is aimed to coordinate inositol metabolism with cellular growth (Majumder et al., 1997). Northern-blot analysis and *in situ*

Table 2. Sequence identity matrix showing the sequence identity on 0-1 scale among eukaryotic protein sequences encoded by MIPS gene.

Seq->	*G. max* 1	*A. thaliana*	*T. aestivum*	*N. tabacum*	*S. indicum*	*B. napus*	*G. max* MIPS1	*G. max* MIPS2	*G. max* MIPS3	*G. max* MIPS4	*P. vulgaris*	*Z. mays*	*A. sativa*	*O. sativa*	*H. vulgare*	*B. juncea*	*C. arietinum*	*V. radiata*	*H. sapiens*	*S. cerevisiae*
G.max 1	ID																			
A.thaliana	0.87	ID																		
T.aestivum	0.89	0.91	ID																	
N.tabacum	0.93	0.91	0.92	ID																
S.indicum	0.92	0.9	0.93	0.94	ID															
B.napus	0.88	0.9	0.94	0.91	0.92	ID														
G.maxMIPS1	1	0.87	0.89	0.92	0.92	0.88	ID													
G.max MIPS2	0.99	0.88	0.9	0.93	0.92	0.89	0.98	ID												
G.maxMIPS3	0.96	0.9	0.91	0.94	0.93	0.9	0.96	0.96	ID											
G.maxMIPS4	0.95	0.89	0.9	0.94	0.93	0.89	0.95	0.95	0.99	ID										
P.vulgaris	0.95	0.88	0.89	0.91	0.91	0.88	0.95	0.96	0.94	0.93	ID									
Z.mays	0.88	0.87	0.89	0.9	0.89	0.88	0.88	0.89	0.89	0.88	0.88	ID								
A.sativa	0.88	0.88	0.89	0.9	0.9	0.89	0.87	0.89	0.89	0.88	0.88	0.94	ID							
O.sativa	0.88	0.86	0.89	0.9	0.89	0.88	0.88	0.89	0.89	0.88	0.88	0.96	0.95	ID						
H.vulgare	0.88	0.87	0.88	0.9	0.89	0.87	0.87	0.88	0.88	0.88	0.88	0.94	0.96	0.95	ID					
B.juncea	0.88	0.86	0.89	0.9	0.89	0.88	0.88	0.89	0.89	0.88	0.88	0.96	0.95	0.99	0.95	ID				
C.arietinum	0.94	0.88	0.89	0.92	0.91	0.89	0.94	0.94	0.94	0.93	0.93	0.86	0.87	0.87	0.86	0.87	ID			
V.radiata	0.96	0.88	0.89	0.92	0.92	0.89	0.96	0.96	0.95	0.95	0.96	0.88	0.88	0.88	0.87	0.88	0.93	ID		
H.sapiens	0.06	0.06	0.05	0.05	0.05	0.05	0.06	0.06	0.06	0.06	0.06	0.05	0.05	0.05	0.05	0.05	0.05	0.06	ID	
S.cerevisiae	0.06	0.07	0.06	0.08	0.07	0.07	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.07	0.07	0.07	0.06	0.06	0.05	ID

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 shoots, roots and flowers (Yoshida et al., 1999). Coelho et al. (2007) in *Phaseolus vulgaris*, reported low concentration of phytate at the initial stages of seed development, coinciding with a period of the most intense seed metabolism followed by a period of high enzymatic activity and gene expression of *MIPS* when a decrease in its specific activity and transcription was detected throughout seed development. Reduced transcript levels of *MIPS1* in other soybean tissues compared to developing cotyledons could be due to low occurrence or absence of inositol or phytic acid biosynthesis in these tissues. The quantification of absolute amounts of cDNA coding for the rice Ins(3)P synthase, RINO1, by real-time RT-PCR revealed the unique spatial expression patterns of *MIPS* genes (Suzuki et al., 2007). In soybean, previous expression studies via Northern blot analysis and semi-quantitative RT-PCR concluded that *MIPS1* isoform was highly expressed in seeds (Hegeman et al., 2001; Kumar et al., 2012). High degree of similarity at amino acid sequence level (Figure 1) and maximum homology at nucleotide level (Figure 2a) among *MIPS* isoforms makes it essential to confirm the organ specificity of each isoform and their temporal and spatial expression profiles so as to specifically target the site and stage for genetic manipulation to obtain low seed phytate phenotypes. Previous expression studies had revealed that *MIPS1* expression was maximum in developing seeds of about 2 to 4 mm by Hegeman et al. (2001) and 6 to 8 mm size stage by Chappell et al. (2006). In the present study however the highest transcript levels of *GmMIPS1* were observed in seeds of 4 to 6 mm size stage. This difference in the peak expression may be attributed to the overlap in the seed

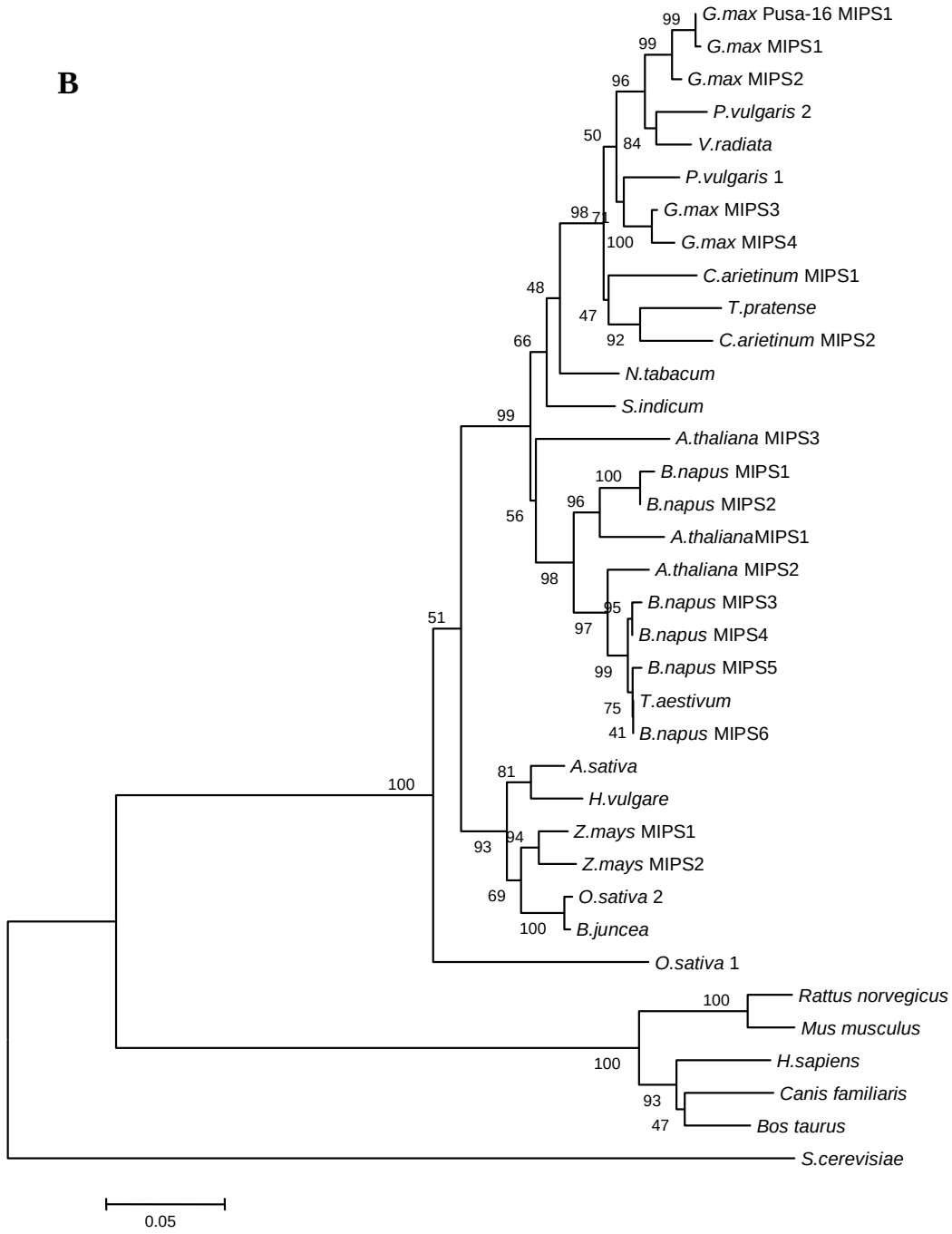

Figure 2. Continued.

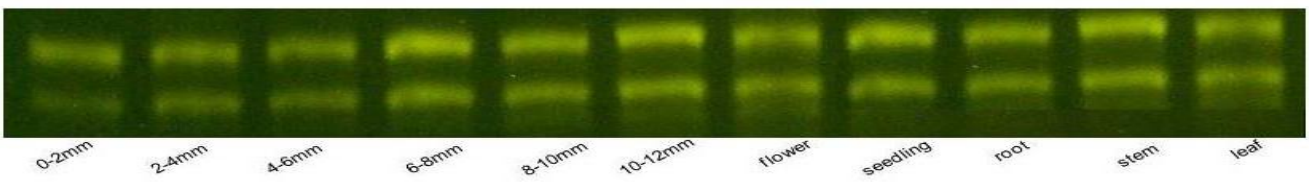

Figure 3. Total RNA from organ tissues and developing seeds of soybean.

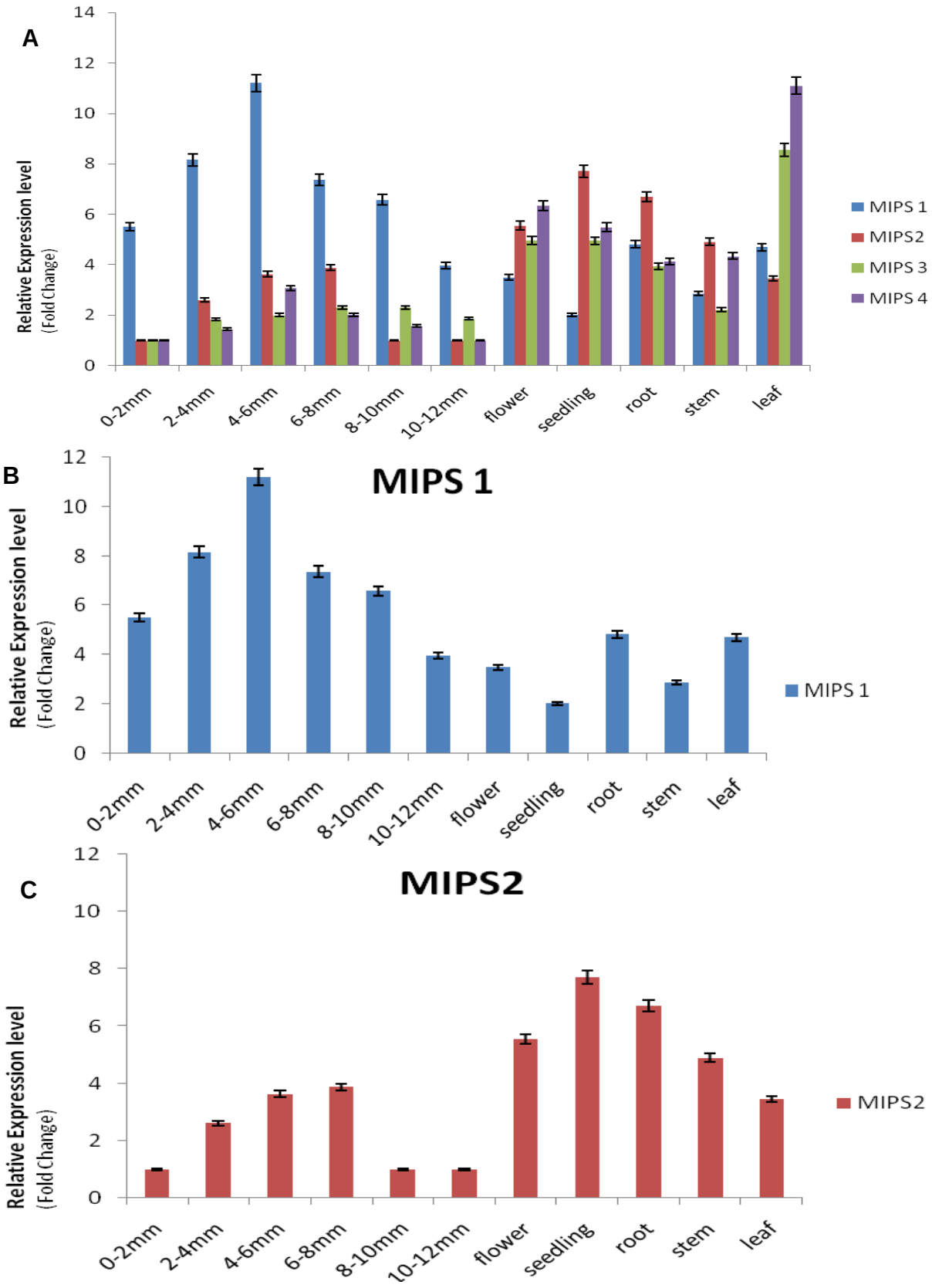

Figure 4. A. Relative expression of soybean *MIPS* genes in developing seeds and organ tissues. B, C, D, E are individual expression profile of *MIPS1, MIPS2, MIPS3, MIPS4* gene.

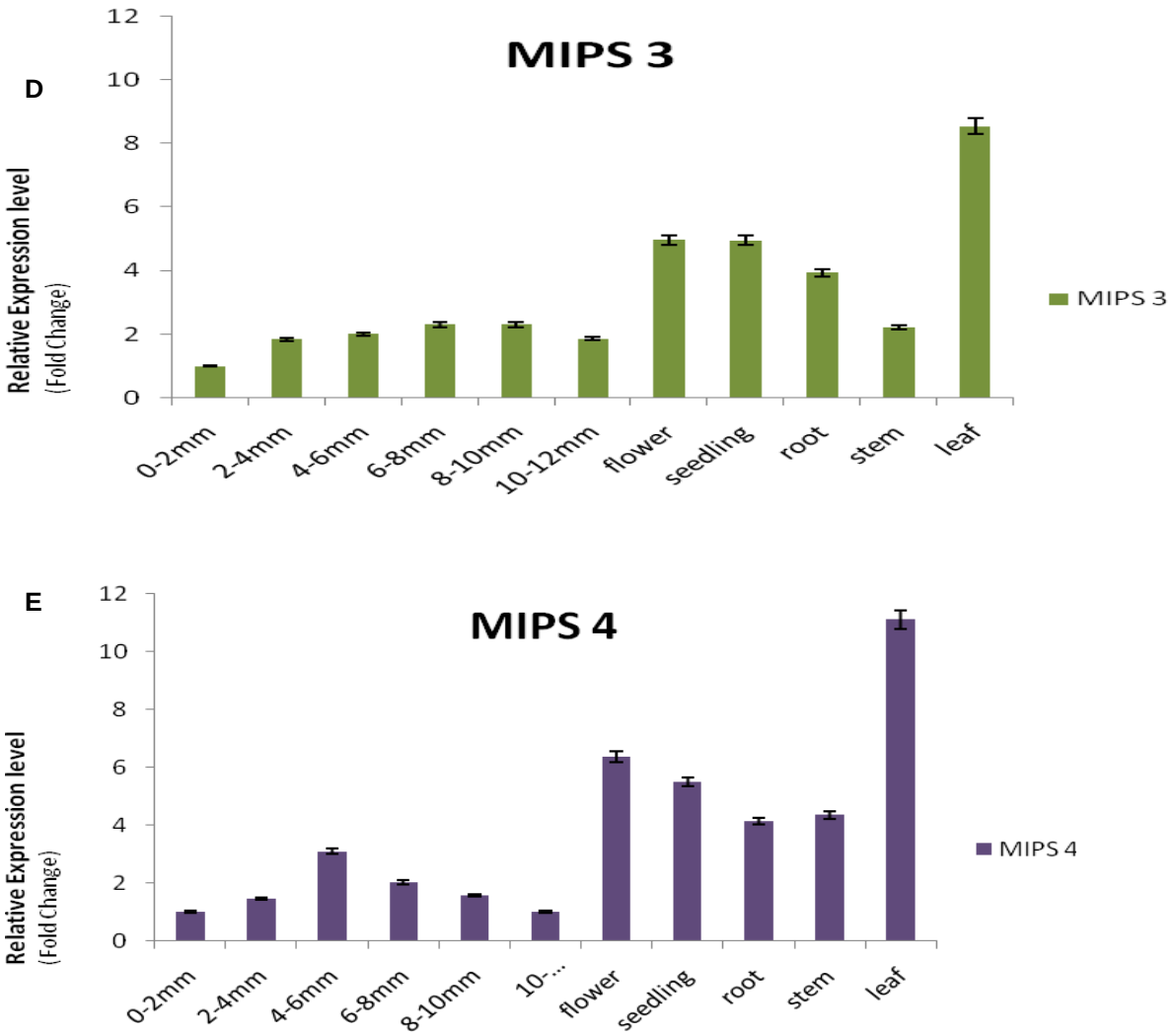

Figure 4. Continued.

sizes during seed collection. This study also indicated that the *MIPS1* transcripts were either not expressed or minimally expressed during the later stages of seed development (10 mm to 12 mm). Overall the expression profile of the *MIPS* gene observed in the present study was consistent with the previous results of Chappell et al. (2006) where *MIPS*1 dominated the expression in developing seeds and *MIPS*2, *MIPS*3 and *MIPS*4 in other organ tissues. The expression analysis clearly confirmed that despite of high degree of sequence conservation among the *MIPS* isoforms, *MIPS*2, *MIPS*3 and *MIPS*4 were differentially expressed in the organ tissues and the regulatory control of the gene had probably diverged to allow differential regulation in different tissues to enable the coordination of inositol metabolism with cellular growth as hypothesized by previous works (Abreau and Aragao, 2007; Chiera and Grabau, 2007; Mitsuhashi et al., 2008). Further to understand the molecular basis of differential expression of the four *MIPS* genes, the complete genomic sequences of all the four isoforms have been determined and it has been reported that they share a common intron/exon structure (Chappell et al., 2006) with a high degree of conservation at both the sequence as well as structural level. Differential expression of these genes has also been explained by their sequence BLAST with the complete soybean genome, sequenced by Schmutz et al. (2010) and complex genome duplication events with nearly 75% of the genes present in multiple copies in the soybean genome were reported. *MIPS* sequence BLAST with complete genome revealed that *MIPS1* had 100% coverage on chromosome 18 and 91% identity on chromosome 10, 5 and 8. *MIPS2* gene was found to have 99% coverage and identity on chromosome 18 and 95% identity on chromosome 9. *MIPS3* had 99% identity on chromosome 5 and also 96% on chromosome 3 while

MIPS4 had 99% identity on chromosome 8. These scattered gene copies throughout the genome may contribute for their differential expression and their duplication may make it difficult, though not impossible, to target the genes necessary to improve soybean characteristics such as seed size, oil content or yield and low phytate seeds.

Conclusion

MIPS, the evolutionarily conserved enzyme throughout eukaryotes, on multiple alignment showed very high identity scores between the four isoforms alongwith the four conserved domains in their amino acid sequences. The results demonstrate distinct dynamic expression patterns of the four *MIPS* genes indicating a possibility of differential expression of each gene to perform specific physiological functions. Amongst the four *MIPS* gene isoforms, *MIPS1* showed predominant transcript levels in the early developing seeds making it a suitable target for down regulation using small RNA-mediated gene silencing tools to reduce the phytate levels in seeds and enhance nutritional value of the transgenic soybean without affecting the critical aspects of inositol metabolism in other tissues of the plant.

ACKNOWLEDGEMENTS

Financial support provided by Indian Agricultural Research Institute, New Delhi, in the form of SRF is duly acknowledged.

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