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Characterisation of structural genes involved in phytic acid biosynthesis in common bean (*Phaseolus vulgaris* L.)

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Abstract Phytate (*myo*-inositol hexa*kis*phosphate), the major form of phosphorous storage in plant seeds, is an inositol phosphate compound poorly digested by humans and monogastric animals. A major goal for grain crop improvement is the reduction of its content in the seed to improve micronutrient bioavailability and phosphorus utilisation by humans and non-ruminant animals, respectively. We are interested in lowering phytic acid in common bean seed and to this goal we have undertaken a two-strategy approach: the isolation of mutants from an EMS mutagenised population (Campion et al. 2009) and the identification of genes coding for candidate enzymes involved in inositol phosphate metabolism for future targeted mutant isolation and/or study. In this paper we report data referred to the second approach and concerning the isolation and genomic organisation of *Phaseolus vulgaris* genes coding for *myo*-inositol 1-phosphate synthase (*PvMIPSs* and *PvMIPSv*), inositol monophosphatase (*PvIMP*), *myo*-inositol kinase (*PvMIK*), inositol 1,4,5-tris-phosphate kinase (*PvIPK2*), inositol 1,3,4-triphosphate 5/6-kinase (*PvITPKα* and *PvITPKβ*) and inositol 1,3,4,5,6 penta*kis*phosphate 2-kinase (*PvIPK1*). All these genes have been mapped on the common bean reference genetic map of McClean (NDSU) 2007 using a virtual mapping strategy. Bean markers, presumably associated to each gene of the phytic acid pathway, have also been identified. In addition, we provide a picture of the expression, during seed development, of the genes involved in phytic acid synthesis, including those such as *MIK*, *IMP* and *IPK2*, for which this information was lacking.

Keywords Gene expression · *Myo*-inositol hexa*kis*phosphate · Nutritional quality · Phosphorous · Phytate · Seed

Abbreviations

DAF	Days after fertilisation
DAG	Days after germination
IMP	Inositol monophosphatase
Ins(3)P_1	1D-myo-inositol 3-phosphate
InsP_3	Inositol triphosphate

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InsP_4	Inositol tetra*kis*phosphate
InsP_5	Inositol penta*kis*phosphate
InsP_6	Inositol hexa*kis*phosphate
IPK1	Inositol 1,3,4,5,6 penta*kis*phosphate 2-kinase
IPK2	Inositol 1,4,5-tris-phosphate kinase
ITPK	Inositol 1,3,4-triphosphate 5/6-kinase
lpa	Low phytic acid
MIK	*myo*-inositol kinase
MIPS	*myo*-inositol 1-phosphate synthase
P_i	Inorganic P
PtdIns	Phosphatidylinositol
PtdIS	Phosphatidylinositol synthase

Introduction

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, InsP_6), beside being the storage form of phosphorus in seeds, is also a major seed antinutrient. In fact it contributes to reduce zinc and iron bioavailability of in the small intestines of humans and monogastric animals when legumes and grains are eaten, and this can lead to serious alimentary deficiencies, typical of humans living in the developing countries where staple foods are mainly seed-based (Sandberg 2002). Phytic acid also contributes substantially to environmental pollution, since non-ruminant animals are unable to metabolize it, and the undigested phytic acid promotes water eutrophycation. Thus, the negative effects of InsP_6 on nutrition and the environment are the main reason for developing low-InsP_6 crops (Abelson 1999; Raboy 2006).

In the last decade a great deal of interest has emerged in the possible health-beneficial roles of seed dietary phytic acid and several articles have reported the role of InsP_6 in cancer prevention and control of experimental tumour growth, progression, and metastasis. In addition, InsP_6 possesses antioxidant activity and other significant benefits for human health, such as the ability to enhance immune system, prevent pathological calcification and kidney stone formation, lower elevated serum cholesterol, and reduce pathological platelet activity (reviewed by Vucenik and Shamsuddin 2006; Graf et al 1987; Grases et al. 2008; Zhang et al. 2005).

On the light of these data, any manipulation of InsP_6 content in the seed, either directed towards increasing or lowering InsP_6, requires knowledge of the key enzymes involved in its biosynthetic pathway.

A simple two-step *de novo myo*-inositol (Ins) biosynthetic pathway is shared by all organisms. The Ins ring is provided only through the conversion of glucose 6-phosphate to 1D-*myo*-inositol 3-phosphate (Ins(3)P_1), carried out by D-*myo*-inositol 3-phosphate synthase (MIPS, EC 5.5.1.4) (Loewus and Murthy 2000). The second step is the hydrolysis of Ins(3)P_1 to Ins and P_i (inorganic P) catalyzed by inositol monophosphatase (IMP, EC 3.1.3.25). This reaction is reversed by the action of *myo*-inositol kinase (MIK, EC 2.7.1.6.4). The finding of *lpa* mutants defective in MIPS and MIK activities (Hitz et al. 2002; Shi et al. 2005; Kim et al. 2008) demonstrates the importance of these enzymes for phytic acid synthesis and accumulation in seeds. Conversely, no *lpa* mutants in IMP function have been described so far; however, Fu et al. (2008) reported that *IMP* expression is reduced in developing seeds of barley *lpa* mutants that accumulate Ins, thus suggesting that the control of Ins levels might be important for "feeding" the phytic acid pathway.

Phosphorylation of the of *myo*-inositol ring may occur either by a "lipid-independent" pathway, entirely independent of inositol lipid synthesis, or by a "lipid-dependent" pathway or via a combination of the two (Stevenson-Paulik et al. 2005). In the "lipid-dependent" pathway *myo*-inositol is converted to phosphatidylinositol (PtdIns) by a phosphatidylinositol synthase (PtdIS), PtdIns is then sequentially phosphorylated to produce PtdIns(4,5)P_2. This molecule is the substrate of a PtdIns-specific phospholipase C activity that releases Ins(1,4,5)P_3, a molecule central to signal transduction (York et al. 1999; Odom et al. 2000; Fujii and York 2005; Seeds et al. 2004). In the "lipid-independent" pathway *myo*-inositol phosphate is sequentially phosphorylated to lead to the formation of phytic acid (reviewed by Raboy and Bowen 2006). These sequential phosphorylation steps involve at least three type of inositol kinases: inositol 1,4,5-trisphosphate kinase (IPK2, EC 2.7.1.151 or EC 2.1.7.140), inositol 1,3,4-trisphosphate 5/6-kinase (ITPK, EC 2.7.1.134) and inositol 1,3,4,5,6 pentakisphosphate 2-kinase (IPK1, EC 2.7.1.158). The involvement of these inositol

kinases in InsP_6 synthesis is supported by the isolation of corresponding *lpa* mutants (Shi et al. 2003; Stevenson-Paulik et al. 2005).

Our main aim is to lower phytic acid amount in common bean (*Phaseolus vulgaris* L.) seed and towards this goal we have undertaken a two-strategy approach: the isolation of mutants from an EMS mutagenised population (Campion et al. 2009) and the identification of genes coding for candidate enzymes involved in inositol phosphate metabolism for future targeted mutant identification. Here we report results of this second approach regarding the isolation and molecular characterisation of common bean structural genes important for phytic acid synthesis in the seed. We used bioinformatic tools to identify and map the bean homologues coding for *myo*-inositol 1-phosphate synthase, inositol monophosphatase, *myo*-inositol kinase, inositol 1,4,5-*tris*-phosphate kinase, inositol 1,3,4-triphosphate 5/6-kinase and inositol 1,3,4,5,6 penta*kis*phosphate 2-kinase. We also present data on the genomic organisation of these genes and on their expression during seed development as well as in other plant tissues.

Materials and methods

Plant material

Common bean plants (cv. Taylor's Horticultural and breeding line 905, Campion et al. 2009) were grown in a phytotron under a 16/8 h light/dark photoperiod, with temperatures set at 25/18° day/night and relative humidity between 60 and 70%.

Seeds and pods were collected from bean plants at different developmental stages over a period of several Days After Flowering (DAF). At 4–6 DAF only pods could be collected while after 12 DAF the cotyledons were sufficiently developed to allow the removal of the seed tegument and the embryo. Leaves, roots and epicotyls were sampled at 8 or 18 days After Germination (DAG). All collected samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Sequence analyses, genes and cDNAs isolation

Genes related to the biosynthesis of phytic acid in *P. vulgaris* were identified by searching the public database at the NCBI website (http://www.ncbi.nlm.nih.gov) using the tBLASTx program (Altschul et al. 1997). Genes from *Zea mays* L., *Arabidopsis thaliana* (L.) Heynh. and *Glycine max* (L.) Merr. were used as query against plant nucleotide and EST databases. Nucleotide sequences were multialigned using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Primers for PCR were designed based on conserved regions found in the resulting alignments (Table IS reports primers and annealing temperatures used for target genes isolation). PCR amplifications were performed using leaf genomic DNA or cDNA from developing cotyledons (12 DAF) or leaf (only for *PvMIPSv* isolation) (see Table IS for more details). cDNAs were obtained from total RNA using the reverse transcriptase Superscript II (Invitrogen), according to the protocol suggested by the manufacturer. To obtain full-length cDNA sequences, 3′-rapid amplifications of cDNA ends (RACE)-PCR were carried out with the 3′-RACE system (Invitrogen), using a forward-specific primer in combination with AUAP (Invitrogen).

Multiple alignment of deduced amino acid sequences as well as the related neighbor-joining branched tree were done using Clustal W software at http://align.genome.jp/.

All the genomic and cDNA sequences isolated were deposited in the EMBL Nucleotide Sequence Database (accession numbers are reported in Table IS).

DNA and RNA isolation and blot analysis

For Southern hybridization, 5 μg of genomic DNA, extracted from leaf as described by Dellaporta et al. (1983), were digested with *Eco*RI and *Hind*III, respectively, resolved on a 0.8% agarose gel and transferred to a nylon membrane (Hybond^{TM} N+, GE Healthcare). Total RNA was isolated from leaves, immature pods or cotyledons as described by van Tunen et al. (1988). Ten microgram of RNA from each sample were resolved on 1.2% (w/v) agarose/formaldehyde/MOPS gel and transferred to a nylon membrane (Hybond^{TM} N+, GE Healthcare).

The isolated cDNA sequences coding for the genes of the phytic acid pathway were labelled with α-[^{32}P]-dCTP using a random primer DNA labelling kit (Fermentas), according to the manufacturer's instructions, and used for membrane hybridisations as

described in Sparvoli et al. (1994). Membranes were washed at 0.1× SSC, 0.5% (w/v) SDS at 65°C.

In silico mapping

Bean genes of phytic acid pathway were mapped onto the soybean genome by "virtual Southern blot" based on similarity searches (Salse et al. 2004) using BLASTn tools with their standard servers' settings (http://www.phytozome.net/search.php?show=blast&targetType=genome&method=Org_soybean). For each bean gene the corresponding cDNA was used as query (Table 1 column I). The identified genomic region containing the soybean hortologous genes (Table 1 columns II, IV) was then analysed at the web site http://soybase.org/gbrowse/cgi-bin/gbrowse/gmax1.01 to look for anchored bean markers surrounding the soybean hortologous gene. The position of each marker was verified on the common bean reference genetic map of McClean (NDSU) 2007, at the Legume Information System web site using the cMAP software (http://lis.comparative-legumes.org/cgi-bin/cmap/viewer?changeMenu=1). Additional bean markers proximal to the first one were placed on the soybean genome in order to identify the closest one linked to the soybean homolog of each gene. An *E* value of 10^{-10} was adopted to claim a significant match between bean marker sequence and the soybean genome. In cases of hits with $10^{-10} < E$ value $< 10^{-3}$ these were also considered to be syntenic with the soybean genome when consistent with the other markers' synteny results. Syntenous regions were defined based on the number and relative order of markers supporting colinearity with the common bean reference map of McClean (NDSU) 2007.

Semi-quantitative RT-PCR and quantitative real-time RT-PCR

For semi-quantitative RT-PCR the pairs of primers K1-574F and K1-495, K3-343F and K3-EndR, K3-3-97F and K3-3-EndR, were used for amplifying *PvIPK1*, *PvITPKα* and *PvITPKβ* genes, respectively (Table IS). For quantitative real-time RT-PCR, primers were designed using the ABI-PRISM Primer Express™ Software V2.0 (Applied Biosystems) (gene specific pairs of primers are reported in Table IIS).

Total RNA, extracted as above, was treated with DNAse I by incubating 10 µg of RNA with 2 U of DNAse I (Roche) at 37°C for 30 min and then heat inactivated for 15 min at 75°C. cDNA was produced from 1 µg of total RNA using the "High Capacity cDNA Archive kit" (Applied Biosystems) according to the manufacturer's protocol (RT step). Each reaction for semi-quantitative RT-PCR was performed in 20 µl containing 10 µl of 2× GoTaq Master Mix (Promega), 0.4 µM each of forward and reverse primers and 50 ng of cDNA. The PCR amplification program was: step 1: 94°C 5 min; step 2: 94°C 30 s, 55°C 30 s, 67°C 1 min (we used 25 cycles for *18S rRNA* and 35 cycles for *PvIPK1*, *PvITPKα* and *PvITPKβ* genes); step 3: 67°C 10 min. Control RNA samples were run without the RT step to confirm the absence of any genomic contamination.

Quantitative real-time RT-PCR runs were performed in triplicate in 96-well optical plates using an ABI 7300 Sequence Detection Systems (Applied Biosystems). Each reaction was in 25 µl containing 11.25 µl of 2.5× Real Master MIX/20X SYBR Solution (Eppendorf), 0.5 µM each of forward and reverse primers and 7.5 ng of cDNA. For the 18S rRNA detection (reference gene), 0.1 ng of cDNA were used. The PCR amplification program was: step 1: 50°C 2 min; step 2: 95°C 10 min; step 3 95°C 15 s, 60°C 1 min 95°C repeated for 40 cycles; step 4: 95°C 15 s, 60°C 30 s, 95°C 15 s. Control RNA samples were run without the RT step to confirm the absence of any genomic contamination.

PCR efficiencies of target and reference genes were determined by generating standard curves. For each target gene, relative transcript levels were normalized to the *18S rRNA* reference gene level and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) following indications described in the SDS ABI 7300 User manual.

Results

Identification and characterization of structural genes of the phytic acid biosynthetic pathway

myo-inositol 1-phosphate synthase

In bean two different MIPS protein forms have been reported: one is specifically expressed in developing seeds while the other one is localized in the plastids

Table 1 List of the closest bean markers (column V) to the bean genes of the phytic acid pathway (column I)

I Pv gene	II Soybean blast (Phytozome)	III *E* value	IV Gene position on soybean genome	V Closest bean marker (GB: ID number)	VI *E* value	VII Bean chromosome McClean (NDSU) 2007	VIII Marker position on soybean genome	IX Distance from soybean gene (bp)
FN356963-PvMIK	Glyma02g39160	2.4e-117	Gm02:44428397..44429410	g2151 (GB: DX823153)	2.9e-101	Pv8 (177 cM)	Gm02:44573730..44575276	145,866
	Glyma14g37260	7.1e-130	Gm14:46546346..46549908	g2151 (GB: DX823153)	3.7e-85	Pv8 (177 cM)	Gm14:46713891..46715343	165,435
FN356965-PvIMP	Glyma09g01380	4.5e-39	Gm09:846198..851195	g2435 (GB: ED510149)	1.1e-3	Pv6 (64 cM)	Gm09:852326..854136	1,131
	Glyma15g12230	7.6e-44	Gm15:9045902..9051034	g2435 (GB: ED510149)	7.3e-5	Pv6 (64 cM)	Gm15:9052173..9053987	1,139
AJ853494-PvMIPSs	Glyma18g02210	2.9e-97	Gm18:1364774..1367704	g1367 (GB: DX823521)	1.4e-114	Pv1 (36 cM)	Gm18:1156101..1157096	210,608
FN356964-PvMIPSv	Glyma05g31450	5.0e-114	Gm05:36578952..36583377	g1247 (GB: DX823103)	3.3e-98	Pv2 (61 cM)	Gm05:36611513..36614291	28,136
	Glyma08g14670	1.8e-104	Gm08:10680522..10685058	g1247 (GB: DX823103)	5.4e-137	Pv2 (61 cM)	Gm08:10717457..10720172	32,399
FN357286-PvITPKα	Glyma05g02250	1.3e-76	Gm05:1663200..1663763	g686 (GB: DX82322)	1.4e-105	Pv3 (60 cM)	Gm05:2263687..2264365	599,924
	Glyma17g09680	0	Gm17:7169985..7171100	g2371 (GB: DX823162)	1.9e-133	Pv3 (78 cM)	Gm17:6172758..6176966	993,019
FN357286-PvITPKβ	Glyma04g09290	1.3e-72	Gm04:7456010..7457950	g1107 (GB: DX823834)	5.8e-90	Pv9 (82 cM)	Gm04:7244444..7256547	199,463
	Glyma06g09430	2.0e-71	Gm06:6952780..6954820	g1107 (GB: DX823834)	1.0e-97	Pv9 (82 cM)	Gm06:6774253..6774807	177,973
FN356968-PvIPK2	Glyma12g36970	7.3e-23	Gm12:39968163..39973000	g188 (GB: ED544197)	1.1e-18	Pv11 (135 cM)	Gm12:39999966..40000030	26,966
FN356967-IPK1	Glyma04g03240	8.8e-152	Gm04:2371335..2378215	g544 (GB: DX823745)	8.9e-96	Pv9 (112 cM)	Gm04:2432156..2433080	53,941
	Glyma06g03310	2.1e-146	Gm06:2336471..2342870	g544 (GB: DX823745)	5.1e-14	9 (112 cM)	Gm06:2385377..2385450	42,507

Markers were identified using the virtual mapping approach. Column III: *E* values from the BLAST search on soybean genome using the Pv gene as query. Column VI: *E* values from the BLAST search on soybean genome using the bean marker as query. Column IX: distance between the soybean gene (column IV) and the closest bean marker (column VIII)

(Johnson and Wang 1996). In addition, the sequence of a *MIPS* cDNA (*PvMIPSr*, GenBank accession U38920) expressed in roots has also been reported (Wang and Johnson 1995). Surprisingly, this sequence is very similar (99% of identity) to the Arabidopsis *AtMIPS1* gene (NCBI Reference Sequence NP_195690).

In order to isolate the gene coding for the MIPS form expressed in developing seeds we performed RT-PCR on cDNA derived from developing common bean seeds using pairs of primers designed on the basis of available bean EST sequences and on the partial genomic common bean *MIPS* (GenBank accession AF282263) present in the GenBank database (www.ncbi.nlm.nih.gov). A full length cDNA of 1,736 bp (called *PvMIPSs*) was isolated and shown to contain an open reading frame of 1,533 bp encoding for a polypeptide of 510 amino acids. When leaf genomic DNA was used as the template for PCR, two types of *MIPS* genes were isolated: one is 2,671 bp long, contains nine introns and its transcript was 100% identical to *PvMIPSs* isolated from developing cotyledons cDNA. The other gene shares a high degree of sequence identity (88%) with *PvMIPSs*, from which it differed mainly for the length of the third intron (Fig. 1). Analysis of the sequences in the bean EST database, together with expression analyses by real-time RT-PCR (Fig. 2) showed that this second gene is expressed in several plant organs, but is poorly represented in developing seeds. It was therefore referred to as *PvMIPSv* (*MIPS* vegetative) to differentiate it from the *PvMIPSs* (*MIPS* seed). The phylogenetic tree derived from the multiple alignment of the deduced amino acid sequences of the bean genes together with those derived from soybean and other species' *MIPS* genes confirmed that *PvMIPSs* clusters together with

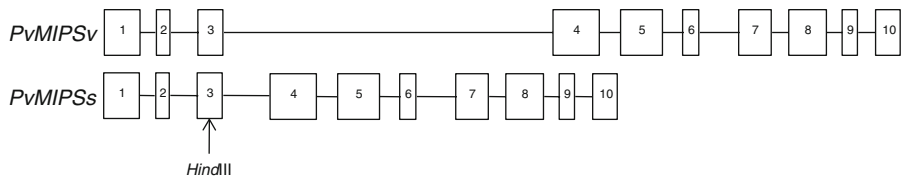

Fig. 1 Intron-exon structure of the bean *PvMIPSs* and *PvMIPSv* genes. Exons are indicated by *boxed numbers*, *lines* represent introns, *upward arrows* indicates sites for restriction enzymes

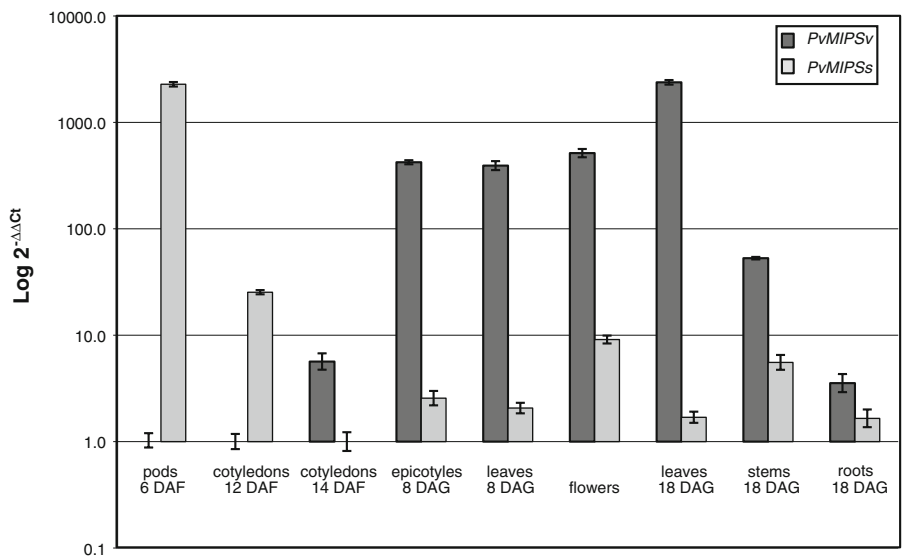

Fig. 2 Quantitative real-time RT-PCR analysis of *PvMIPs* and *PvMIPSv* on cDNAs from different bean plant tissues. Each *bar* represents the mean of three replicates with the indicated standard deviation of the mean. *DAF* indicates Days After Fertilisation; *DAG* indicates Days After Germination

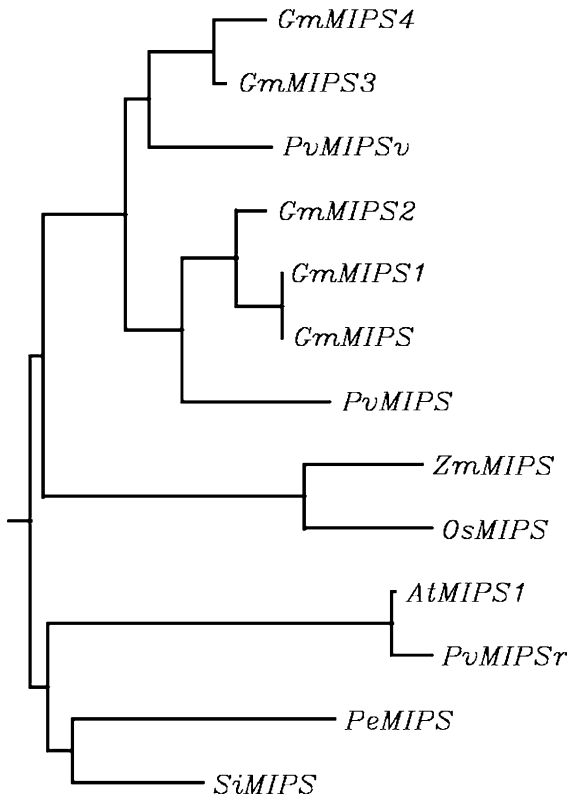

Fig. 3 Phylogenetic tree of MIPS proteins. Accession numbers are: GmMIPS (AAK72098), GmMIPS1 (ABC55419), GmMIPS2 (ABC55420), GmMIPS3 (ABC55421), GmMIPS4 (ABC55422), ZmMIPS (AAG40328), OsMIPS (BAA25729), AtMIPS1 (NP_195690), PvMIPSr (Q41107), PeMIPS (ABF51620), SiMIPS (AAG01148), PvMIPs (CAH68559), PvMIPSv (CAJ15162)

GmMIPS1, which is highly expressed in the cotyledons, while *PvMIPSv* is in the same branch with *GmMIPS3* and *GmMIPS4*, which are expressed in vegetative tissues and poorly, or not at all, in seed tissues (Fig. 3) (Chappell et al. 2006).

The presence of two different *MIPS* genes as well as their copy number was determined by Southern blot hybridization (Fig. 4a). The hybridisation pattern obtained with the two genes clearly indicated the presence of one copy for each *MIPS* gene. In fact, comparing the two blots it was evident that the stronger hybridising fragments obtained with the *PvMIPSs* probe corresponded to the weaker ones recognised by the *PvMIPSv* probe. Moreover, when genomic DNA was digested with *Hind*III and hybridised to the *PvMIPSs* probe, an additional hybridising fragment was detected that corresponded to the 5′ region of the gene which contains a *Hind*III site which is absent in the *PvMIPSv* gene (Fig. 1, 4a).

Inositol monophosphatase (PvIMP)

The bean cDNA coding for IMP (*PvIMP*) was isolated by RT-PCR from developing seed cDNA using pairs of primers designed on the basis of *P. vulgaris* ESTs (GenBank accessions CV532534, CV538392 and FD787042) homologous to *LeIMP1* (GenBank accession AY227666) reported in tomato. The isolated *PvIMP* cDNA is 900 bp long and codes for a polypeptide of 272 amino acids. The amino acid sequence coded by *PvIMP* was aligned to the sequences of other IMP proteins from tomato, barley, *Arabidopsis*, rice and ice plant. Amino acid residues reported by Bone et al. (1992) to be involved in metal or sulphate binding (highlighted residues in Fig. 1S) as well as motifs A (WxI/VDPI/LDxTxxF/Y/IxH/K) and B (WDxxA/GA/GxA/I/LI/L/VA/L/VxxxGA/G) described by Neuwald et al. (1991) (boxed residues in Fig. 1S) were highly conserved among all the sequences, thus suggesting that PvIMP is endowed with inositol monophosphatase activity.

On the basis of the alignment between the *PvIMP* cDNA and the *LeIMP1* genomic sequence, we designed other pairs of primers in order to isolate the corresponding entire bean genomic sequence. The genomic sequence of *PvIMP* was 5,862 bp long and was organised with a 12-exons-11-introns structure. From the comparison of the intron-exon structure of *PvIMP* with *IMP* genes from other species we observed that dicot species share the 12-exons-11-introns structure, while monocots have a 10-exons-9-introns organisation, in which monocot exon 1 and exon 3 are equivalent to dicot exon 2 and exons 4 and 5, respectively (Fig. 5).

The number of *PvIMP* genes present in the bean genome was estimated by Southern blot analysis (Fig. 4b). Digestion of leaf genomic DNA with *EcoRI* and *Hind*III showed the presence of few hybridising fragments. Sites for these two restriction enzymes are present in the *PvIMP* sequence (three for *Eco*RI and one for *Hind*III) and the length of the major *Eco*RI hybridising fragment (about 5,000 bp) together with the number of hybridising fragments obtained with both enzymes is consistent with the hypothesis of the presence of a single copy of the *IMP* gene in bean (Fig. 4b, 5).

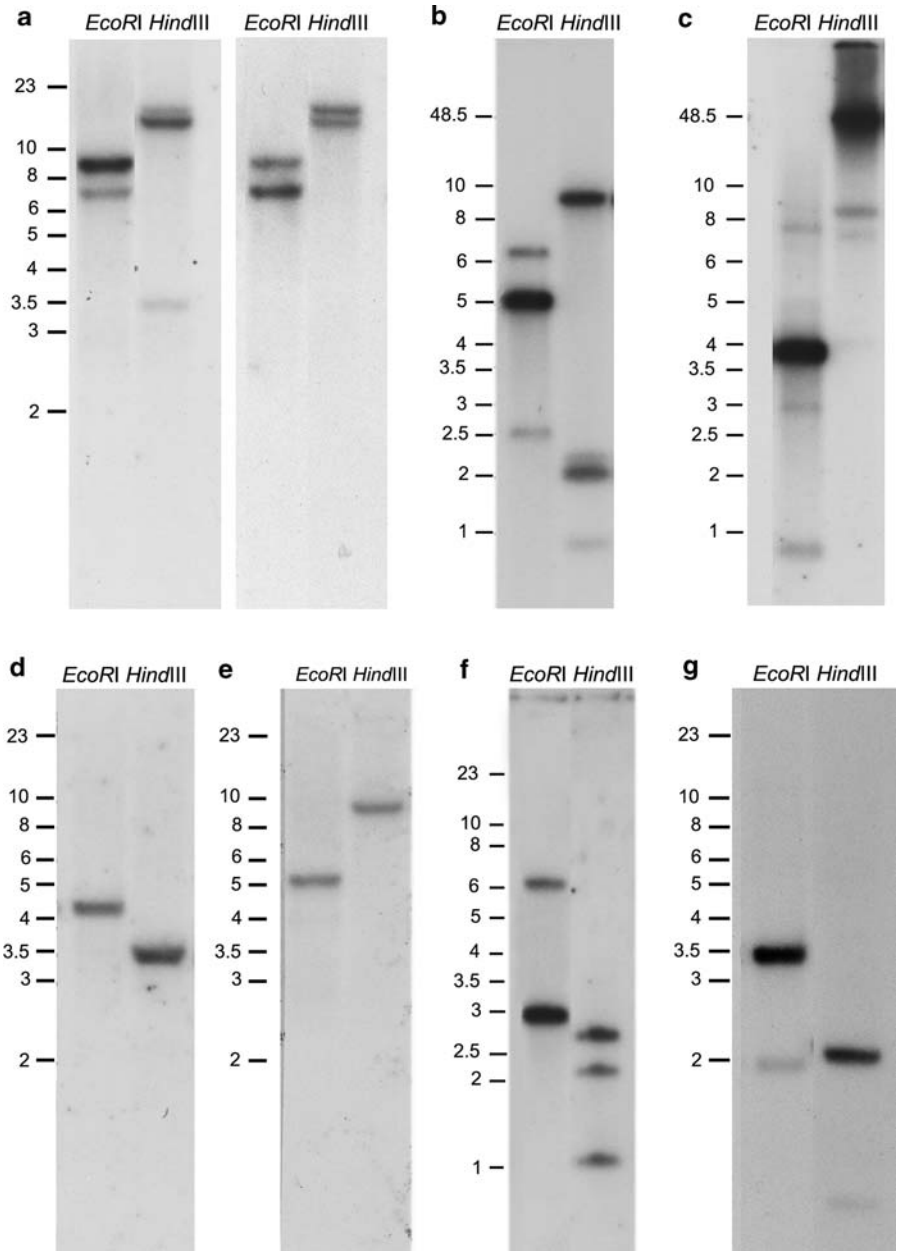

Fig. 4 Southern blot analysis of genes of the phytic acid pathway. Genomic DNA was digested with the restriction enzymes *Eco*RI or *Hind*III. Filters were hybridised to **a** *PvMIPSs* or *PvMIPSv*, **b** *PvIMP*, **c** *PvMIK*, **d** *PvIPK2*, **e** *PvITPKα*, **f** *PvITPKβ,* **g** *PvIPK1*. On the *left side* of each *blot* are reported molecular weights in kb

Myo-inositol kinase

Myo-inositol kinase (MIK) phosphorylates *myo*-inositol to produce Ins(3)*P*. The involvement of this activity in the production of phytic acid has been shown by Shi et al. (2005) who demonstrated that in the maize *lpa*3 the *MIK* gene is disrupted by a Mu transposon insertion. In the *lpa3* mutant the decrease in phytic acid is accompanied by an increase in *myo*-inositol, while *myo*-inositol phosphate intermediates are not present at significant levels. These characteristics suggest that MIK is not just a salvage enzyme for *myo*-inositol recycling, but is an important biosynthetic step of phytic acid pathway (Shi et al. 2005).

Since it was not possible to identify any putative MIK sequence in the bean EST database, we used the

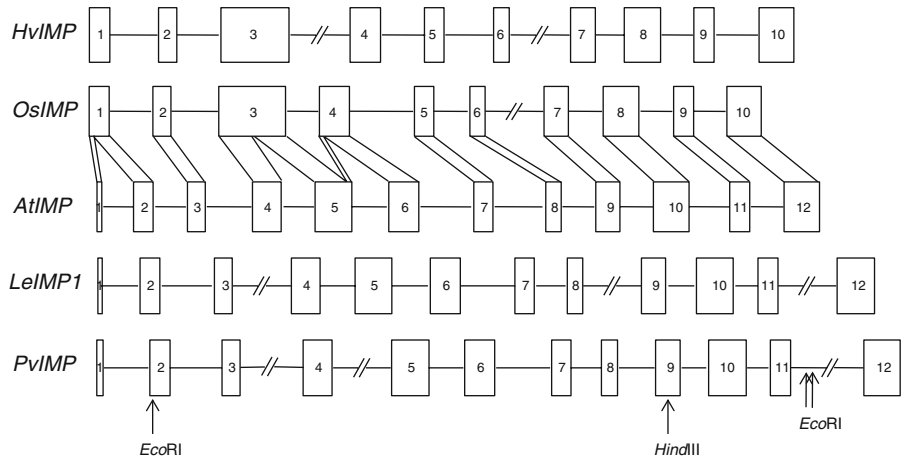

Fig. 5 Comparison of bean *PvIMP* exon-intron structure with that reported for other IMP genes. Exons are indicated by *boxed numbers*, *lines* represent introns, *upward arrows* indicates sites for restriction enzymes. Accession numbers are: *HvIMP* (DQ145527), *OsIMP* (AK071149), *AtIMP* (AY035150), *LeIMP1* (AY227666), *PvIMP* (FN357287)

maize *MIK* (GenBank accession AY772410) as a query to search genomic and ESTs plant databases to find other putative *MIK* sequences in order to build a multiple sequence alignment for primer design. The putative *MIK* sequences used were those of soybean (GenBank accession CS236223), *Lotus japonica* (GenBank accession BW619491), *Medicago truncatula* (GenBank accession EV255698), *Cicer arietinum* (CK148972) and those already reported in rice, maize and *Arabidopsis* by Shi et al. (2005). The RT-PCR was conducted on developing seed cDNA and the amplification product (715 bp) was sequenced. We were able to obtain only a portion of the *PvMIK* sequence corresponding to a polypeptide of 238 amino acids that share an overall amino acid identity of 83, 58, 57 and 56% with MIK proteins of soybean, rice, *Arabidopsis* and maize, respectively.

In maize MIK is coded by a single-copy gene containing only one intron, and also in *Arabidopsis* a single *MIK* sequence with only one intron is present. The *PvMIK* cDNA corresponds to the region coded by exon 1 both in *Arabidopsis* and maize, therefore, assuming a similar gene organisation in bean, we can affirm that no *Eco*RI nor *Hind*III restriction sites are present in the *PvMIK* gene, since these enzymes do not cut the cDNA. From Southern blot analysis also the bean genome appears to contain a single copy of the MIK gene, in fact a single hybridising fragment is present in genomic DNA digested with *Eco*RI or *Hind*III (Fig. 4c). Weaker hybridising fragments are also recognised by the *PvMIK* probe, however, we hypothesise that they correspond to pseudogenes or distantly related genes, since the blot was washed in highly stringent conditions (0.1xSSC at 65° for 1 h).

Inositol 1,4,5-tris-phosphate kinase/inositol polyphosphate multikinase

Inositol 1,4,5-*tris*-phosphate kinase, or more appropriately inositol polyphosphate multikinase, is a dual-specificity InsP_3/InsP_4 6-/3-kinase that sequentially generates InsP_5 from InsP_3 (Stevenson-Paulik et al. 2002; Shears 2004; Xia and Yang 2005). The involvement of this enzyme activity in phytic acid biosynthesis has been demonstrated by Stevenson-Paulik et al. (2005) who showed that the loss-of-function obtained by T-DNA insertion in the Arabidopsis *AtIpk2β* gene resulted in an accumulation of intermediate inositol phosphorylated forms and in a seed phytic acid reduction of about 35%.

To look for bean *IPK2* homologues, the *AtIpk2β* gene of *Arabidopsis* was used as query in a tBLASTx search against the soybean EST database. A contig corresponding to the *GmIPK2* was found and aligned with the *AtIpk2β* gene to allow the designing of PCR primers in conserved regions. By RT-PCR on cDNA from developing seeds we isolated a sequence of 1,116 bp, named *PvIPK2*, coding for a polypeptide of 264 amino acids. The alignment of this polypeptide to other plant IPK2 proteins showed a degree of identity

ranging from 72% with the soybean Ipk2 to 48% with the rice sequence (OsIPK2) (Fig. 2S). The PvIPK2 protein contains the PxxxDxKxG conserved InsP_3 binding domain, which is a catalytic site for phosphate transfer from ATP to the inositol ring (Bertsch et al. 2000). The motif [L/M] [I/V]D[F/L][A/G] [H/K] which is considered a putative ATP/Mg^{2+} binding site is also present, together with the "SSLL" domain necessary for enzymatic activity (Saiardi et al. 2001).

The *PvIPK2* gene was intronless as shown by PCR on genomic DNA. Indeed, *Arabidopsis* and rice *IPK2* are also intronless genes. Southern blot analysis showed only one strong hybridising fragment when genomic DNA was digested with restriction enzymes *Eco*RI and *Hind*III. The latter yields a hybridising band of about 3,400 bp, suggesting that *PvIPK2* is a single copy gene (Fig. 4d).

Inositol 1,3,4-triphosphate 5/6-kinase

The family of inositol 1,3,4-triphosphate 5/6-kinases belongs to a larger family of ATP-grasp fold proteins. These also show some functional and structural similarity with inositol polyphosphate multikinases, which include inositol hexa*kis*phosphate kinase and inositol 1,4,5-triphosphate 3-kinase. In plants this type of activity is coded by at least three different types of genes (Suzuki et al. 2007; Sweetman et al. 2007; Stiles et al. 2008), among which only one has been demonstrated to be necessary for phytic acid synthesis in the seed (Shi et al. 2003). We used this last sequence, together with the *Arabidopsis* homologous gene, At5g16760, as a query against the bean EST database. Using the tBLASTx program we found one EST showing 66 and 68% similarity to the maize and *Arabidopsis* sequences, respectively. Primers for PCR were based on this bean EST and allowed the isolation of a PCR fragment of 1,017 bp coding for a polypeptide of 317 amino acids, both from seed cDNA and leaf genomic DNA. The isolated gene, named *PvITPK*α, did not show the presence of any intron and, most likely, is a single copy gene, since from Southern blot analysis with two different restriction enzymes only one hybridising band is detected in high stringency conditions (Fig. 4e).

Although a role in seed phytic acid synthesis has been proven only for the maize *lpa2* gene, other genes coding for inositol 1,3,4-triphosphate 5/6-kinases have been isolated and shown to be expressed in developing seeds (Suzuki et al. 2007; Sweetman et al. 2007; Stiles et al. 2008). Therefore, we decided to look for other members of this family of proteins. The derived amino acid sequences of soybean, rice and *Arabidopsis ITPKs*, together with those from potato and barley, were multiple aligned and the resulting phylogenetic tree confirmed the existence of three types of genes that grouped into three branches (Fig. 6). Soybean genes, belonging to β-branch, as well as *Arabidopsis* and rice genes belonging to γ-branch, were used as query in a tBLASTx search against bean ESTs. Matching ESTs were found only for β-type *ITPK* sequences and used to design primers for RT-PCR on seed cDNA. An amplification product of 658 bp was isolated and shown to correspond to a partial cDNA, named *PvITPK*β, of which the derived amino acid

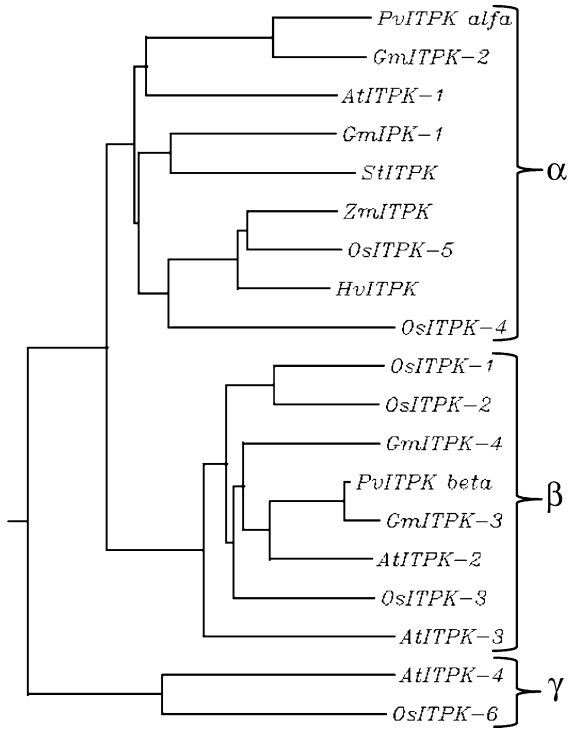

Fig. 6 Phylogenetic tree of ITPK proteins. The three subgroups α, β and γ are indicated. Accession numbers are: GmITPK-1 (ABU93831), GmITPK-2 (ABU93832), GmITPK-3 (ABU93833), GmITPK-4 (ABU93834), AtITPK-1 (NP_197178), AtITPK-2 (NP_849342), AtITPK-3 (ABJ98567), AtITPK-4 (NP_850407), HvITPK (CAL49035), OsITPK-1 (BAF25933), OsITPK-2 (BAF11371), OsITPK-3 (BAF13047), OsITPK-4 (Q84Y01), OsITPK-5 (BAF27322), OsITPK-6 (BAF25603), StITPK (CAM12754), ZmITPK (NP_001105901), PvITPKα (CAP09175), PvITPKβ (CAY10403)

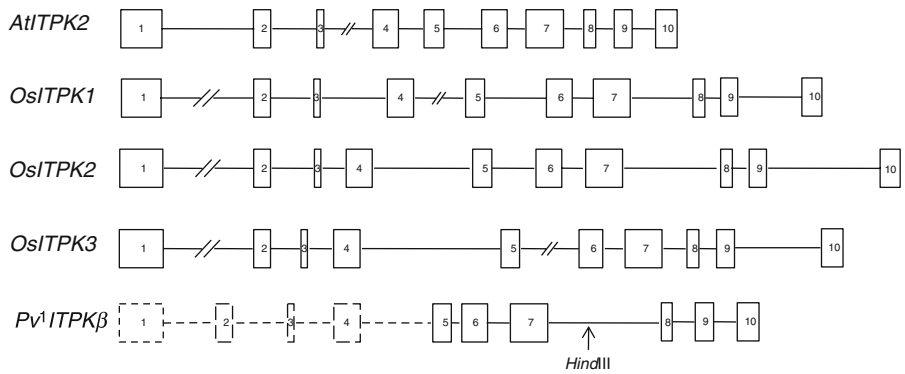

Fig. 7 Comparison of bean *PvITPKβ* exon-intron structure with that reported for other *β*-type *ITPK* genes. Exons are indicated by *boxed numbers*, *lines* represent introns, *upward arrows* indicates sites for restriction enzymes. *Dashed lines* indicate regions not sequenced. Accession numbers are: *AtITPK2* (NM_179011), *OsITPK1* (NC_008403), *OsITPK3* (NC_008396), *PvITPKβ* (FN357285)

sequence was 96% identical to that coded by the *GmITPK3* gene. We also performed PCR amplification on leaf genomic DNA and obtained a sequence of 1,903 bp containing a portion of the *PvITPKβ* gene (Fig. 7). A comparison of the genomic sequences of *β*-type sequences from *Arabidopsis* and barley shows they have 10 exons and 9 introns. The alignment of the bean *PvITPKβ* genomic fragment with these genes indicates that *PvITPKβ* covers a region that spans exon 5 to exon 10 (Fig. 8). All the *β*-type genes have corresponding exons of similar length, while the length of the corresponding introns is more variable, even among genes from the same species (rice).

Southern blot analysis on genomic DNA digested with *Eco*RI or *Hind*III enzymes indicates that *PvITPKα* is a single copy gene, while we cannot exclude the presence of two copies for *PvITPKβ* (Fig. 4f).

Inositol 1,3,4,5,6 pentakisphosphate 2-kinase

The *AtIpK1* gene of *Arabidopsis*, reported to be necessary for the production of phytic acid (Stevenson-Paulik et al. 2005), was used as query against the plant gene database. In fact, we could not identify any bean EST coding for inositol 1,3,4,5,6 penta*kis*phosphate 2-kinase. Full-length cDNAs and genomic DNAs with high similarity (66–82%) to the *Arabidopsis AtIpk1* sequence were found in soybean, rice and maize. In addition, highly homologous ESTs of

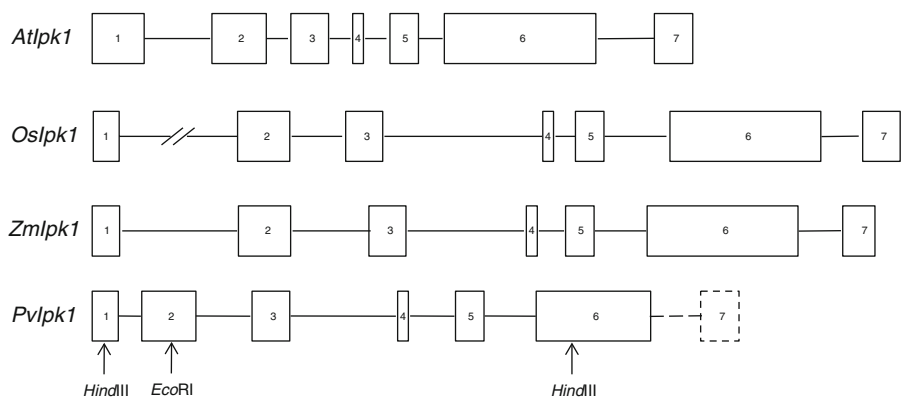

Fig. 8 Comparison of bean *PvIPK1* exon-intron structure with that reported for other *IPK1* genes. Exons are indicated by *boxed numbers*, *lines* represent introns, *upward arrows* indicates sites for restriction enzymes. *Dashed lines* indicate regions not sequenced. Accession numbers are: *AtIPK1* (NP_568613), *OsIPK1* (NP_001054147), Zm*IPK1* (NP_001106063), *PvIPK1* (CAM33431)

soybean, barley, and sorghum were also identified. Multiple sequence alignment of the above identified *IPK*1 sequences revealed several motifs defining conserved regions present in all *IPK*1 members. Then, several pairs of PCR primers were designed along these conserved regions and used for PCR amplification on leaf genomic DNA. A 2,258 bp genomic sequence, named *PvIPK1*, containing an open reading frame of 352 amino acids (from exon 1 to exon 6) was isolated (Fig. 8). The analysis of the exon-intron structure of the *IPK1* genomic clones of *Arabidopsis*, maize, rice and bean indicate that it is conserved among these species, although the length of corresponding introns may be quite different (Fig. 8). The deduced amino acid sequence of the *PvIPK1* gene was aligned with those of soybean, *Arabidopsis*, maize and rice (Fig. 3S) and shown to have a similarity ranging from 83% with the soybean protein to 67% with that of rice. The PvIPK1 protein also contains the conserved motif boxes A, C, E, F and G described by Verbsky et al. (2002), Sweetman et al. (2006) (boxes A-E), and Sun et al. (2007) (boxes F, G).

In order to understand the genomic organization of the *PvIPK1* gene a Southern blot analysis was performed. Under stringent washing conditions (0.1xSSC) one strong and one weaker hybridising fragments were detected using the *PvIPK1* cDNA as probe on genomic DNA digested with *Eco*RI and *Hind*III (Fig. 4g). In the *PvIPK1* genomic sequence *Hind*III cuts twice and the expected hybridization fragments of about 1,900 and 500 bp were detected. Since the *PvIPK1* restriction pattern resulting from Southern hybridisation results is coherent with the size of expected *Hind*III and *Eco*RI fragments, the results indicate the presence of only one copy of the *PvIPK1* gene in the genome of common bean.

In silico mapping of genes of the phytic acid pathway

Taking advantage of the high similarity and synteny between soybean and common bean genomes (Choi et al. 2004) we used an in silico approach to position each gene reported in this paper on bean genetic maps. Soybean has a polyploid genome ($2n = 40$) that is predicted to have undergone duplication after divergence from common bean ($2n = 22$). As expected, we found two hortologous soybean genes for each bean cDNA, the only exceptions being *PvMIPSs* and *PvIPK2* genes for which only one soybean homolog could be identified (Table 1; Fig. 4S). In most cases little rearrangements in the order of bean markers on the corresponding soybean chromosomes were observed (Fig. 4Sa–h).

The results of the comparative mapping analyses allowed the positioning of *PvMIPS*s and *PvMIPSv* on different soybean chromosomes: Gm18 and the two syntenic Gm05 and Gm08, respectively. The subsequent analysis based on adjacent bean anchored markers indicated that the syntenic regions containing these genes are located on bean chromosomes Pv1 and Pv2, respectively (Fig. 4Sa–b). The closest bean marker was at about 210 Kbp in the case of the soybean *MIPSs* gene (Glyma18g02210) and at 28,136 and 32,399 bp in the case of soybean *MIPSv* genes Glyma05g31450 and Glyma08g14670, respectively.

Distances between the closest bean markers and the other soybean genes were in the range of those observed for *MIPS* genes. The only exception was observed for *PvITPK*α, in fact, although the genomic regions surrounding the soybean hortologous were syntenic to the bean chromosome Pv3, the closest bean markers we could find were g686 and g2371 distant 599,924 bp from Glyma05g02250 and 993,019 bp from Glyma17g09680, respectively (Fig. 4Sc). Conversely, in the case of *PvIMP*, which maps on bean chromosome Pv6 (Fig. 4Sd), the marker g2435 was only 1,131 and 1,139 bp from Glyma09g01380 and Glyma15g12230, respectively.

Mapping of *PvIPK2* gene required the use other bean maps together with that of McClean (NDSU) 2007. In fact, the bean marker Bng070, that was 326,417 bp from the soybean Glyma12g36970 and mapped on J linkage group by Vallejos et al. (1992), was not present in the McClean (NDSU) 2007 map (Fig. 4Se). However, using the cMAP software it was possible to align the J linkage group to Pv11 of McClean (NDSU) 2007 map and this allowed the finding of the g188 marker that was much closer (26,966 bp) than the Bng070 to the soybean Glyma12g36970 gene.

Expression of genes involved in phytic acid biosynthesis

In bean seeds phytic acid concentration begins to increase after 16 days after fertilisation (DAF) and reaches a plateau after 26 DAF, while Ins*P* pools

(InsP_3 to InsP_5) peak mainly at 21 DAF (Coehlo et al. 2005), therefore the expression of the genes involved in the phytic acid pathway was monitored at different stages of seed development, ranging from 4 DAF up to 24 DAF. We performed the analyses either by northern blot or, in the case of those genes that were expressed at low levels, by semi-quantitative RT-PCR (Fig. 9).

Maximal expression of *PvMIPS* was observed at the earliest development stages analyzed, i.e. pods at

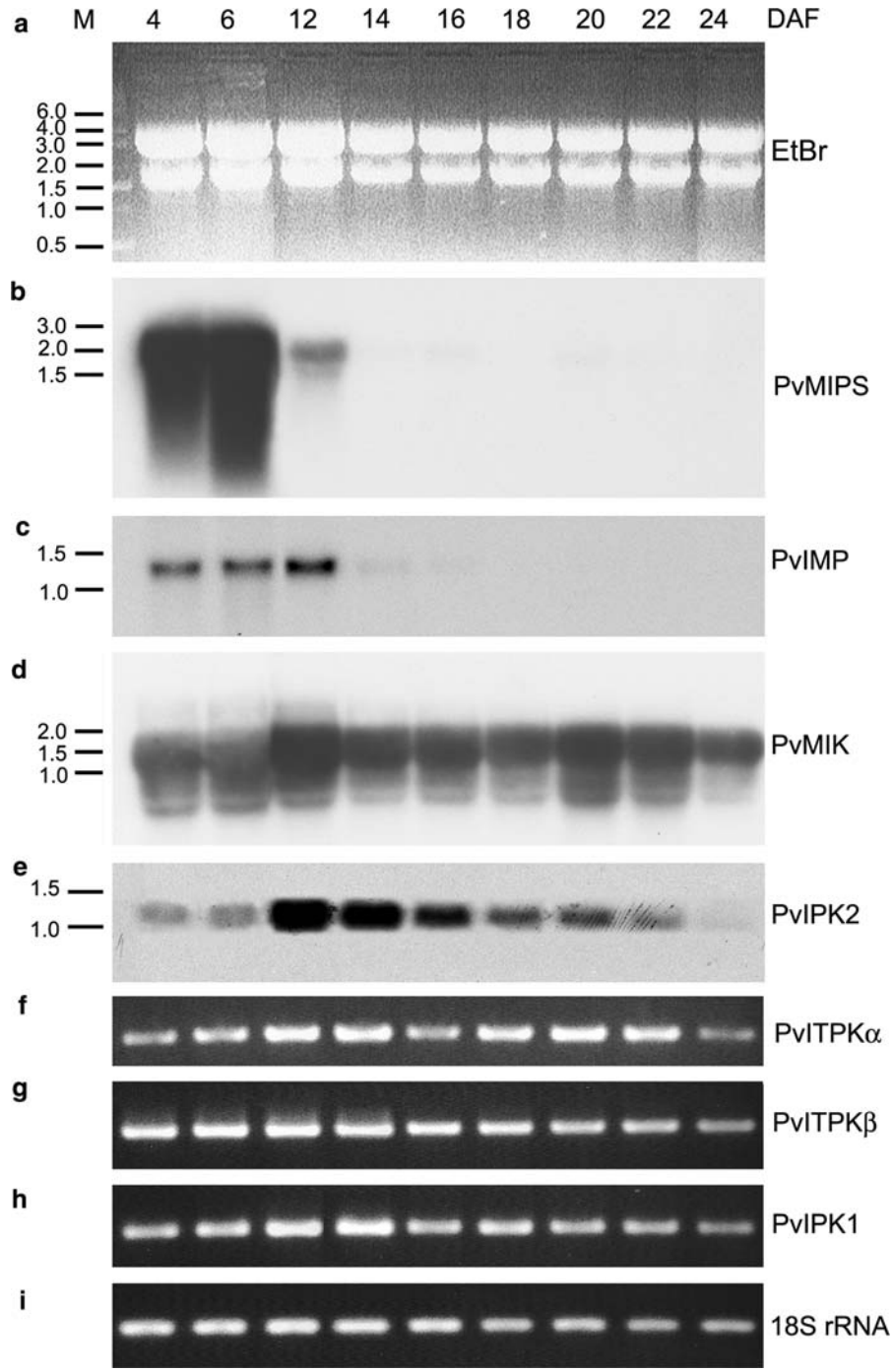

Fig. 9 Expression analysis of genes of the phytic acid pathway during bean seed development. *Panel a*: total RNA before blotting; *panels b–e*: northern analysis using as probes *PvMIPS*, *PvIMP*, *PvMIK* and *PvIPK2*, respectively. *Panels f–i*: semiquantitative RT-PCR of *PvITPKα*, *PvITPKβ*, *PvIPK1*, and 18SrRNA, respectively. *M* indicates molecular weights in kb; *DAF* indicates Days After Fertilisation

4–6 DAF, in which the seed is still almost undetectable and the embryo is at the globular stage. As seed development progressed, *PvMIPS* transcripts decreased to nearly undetectable levels (cotyledons at 14 DAF). Since the two *PvMIPS* genes are highly homologous, in order to establish which one is specifically expressed in the seed we performed a quantitative real-time RT-PCR analysis, using gene specific primers, on the same samples used for northern analysis (pods at 6 DAF and cotyledons at 12 and 14 DAF) and in other plant tissues: epicotyls and young leaves taken from 8 days after germination (DAG) seedlings, leaves, stems and roots from 18 DAG seedlings and flowers (Fig. 2). The results obtained demonstrated that *PvMIPSs* is highly expressed in cotyledons and at much lower levels in flowers as well as in all the other tissues analysed. By contrast, *PvMIPSv* expression is higher in all seedling tissues with the exception of roots where its level is similar to that of *PvMIPSs*.

A clearly regulated expression is also found for *PvIMP* and *PvIPK2* genes during seed development, while the other genes of the pathway do not appear to be significantly regulated during seed development. Their expression was maintained at very similar levels along all the stages analysed, although a very weak reduction can be observed during the latest developmental stages (20–24 DAF) for the *PvITPKβ* gene, while a very faint peak of expression is detectable at 12–14 DAF for the *PvITPKα* gene.

We also decided to investigate the expression of *PvITPKα*, *PvIPK2* and *PvIPK1* in different plant tissues (Fig. 10). The *PvIPK1* gene is expressed at very low levels in all tissues analysed, a result very similar to that observed by Suzuki et al. (2007) in rice. A similar expression was found for the *PvITPKα* gene. On the contrary, *PvIPK2* was highly expressed in developing cotyledons (indeed this was the only gene for which expression could be easily detected by Northern analysis), thus confirming its key role for phytic acid synthesis in this tissue. In the other tissues analysed, *PvIPK2* expression was between 4- and 16-fold lower than that found in cotyledons: the lowest expression was in epicotyls and leaves, while the highest was in flowers.

Discussion and conclusions

In this paper we report the isolation, molecular characterisation and in silico mapping of a full set of structural genes involved in phytic acid synthesis from a single food crop species, *P. vulgaris*.

Mutants impaired in phytic acid accumulation have been reported in several species for almost all the genes described here, however, a direct connection to phytic acid accumulation is still lacking for IMP and ITPK*β*. Inositol monophosphatase catalyzes the dephosphorylation of Ins(3)*P* to produce free *myo*-inositol. No *lpa* mutant with a defective IMP gene has

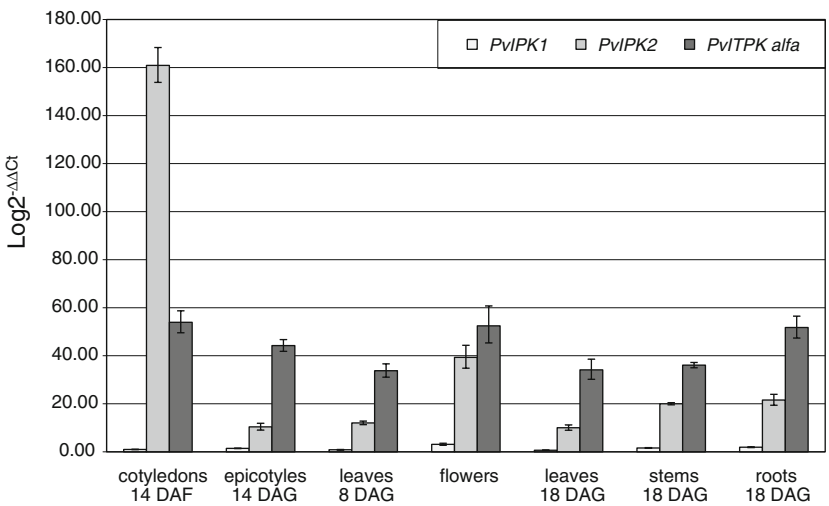

Fig. 10 Quantitative real-time RT-PCR analysis of *PvIPK1, PvIPK2*, *PvITPKα*, on cDNAs from different bean plant tissues. Each *bar* represents the mean of three replicates with the indicated standard deviation of the mean

been reported yet, however, a decrease in IMP-1 expression has been shown in four *lpa* barley mutants (*lpa*1-1, *lpa*2-1, *lpa*3-1 and M955), in which the level of reduction appeared fairly proportional to both phytic acid decrease and *myo*-inositol increase (Fu et al. 2008). However, the role of such type of gene in phytic acid synthesis is still unclear, in fact the protein it codes may be a L-Galactose 1-P phosphatase important to ascorbic acid synthesis, or may be a dual-function as both an L-Gal 1-P phosphatase/IMPase (Laing et al. 2004; Torabinejad et al. 2009). Plant genomes also contain other distantly related "IMPase-like" proteins, more related to prokaryotic IMPases, which very recently have been shown to have IMPase activity (Torabinejad et al. 2009). Therefore, we cannot exclude that these IMPase-like proteins may be more important to inositol supply for phytic acid synthesis then the previously reported IMPase, that was used in this study for *PvIMP* gene identification. However, our finding that *PvIMP* is developmentally regulated during seed maturation together with the fact that *lpa* mutants show a decrease in the expression of this gene proportional to both phytic acid decrease and *myo*-inositol increase (Fu et al. 2008), is a good indication supporting a role of this gene in phytic acid biosynthesis. In addition, we have observed a lower expression of *PvIMP* in developing seeds of bean *lpa*-280-10 mutant compared to wt (manuscript in preparation), thus confirming what reported in other *lpa* mutants.

Our work advances efforts to analyse the complete set of genes and functions important to phytic acid synthesis in seeds. In fact, although the phytic acid pathway has been studied in some detail in several species, i.e. rice, barley, maize, *Arabidopsis* and soybean, each of these species lacks a detailed description of some specific genes of the pathway. For example, in maize no data are available for *IMP* and *IPK2* genes, while in rice, for which all the genes of the pathway have been identified, data are lacking for *MIK* gene copy number and *MIK*, *IMP* and *IPK2* expression during seed development. Here, we demonstrate that beside *MIPS* also *IMP* and *IPK2* expression are regulated during seed development, underlining the key role of these genes in phytic acid biosynthesis, a result that is supported by the finding that these genes are down-regulated in the bean *lpa*-280-10 mutant (manuscript in preparation). Moreover, we provide for the first time a clear picture of the expression during seed development of the genes involved in phytic acid synthesis, including those such as *MIK*, *IMP* and *IPK2*, for which this information was lacking.

MIPS enzyme is necessary for *myo*-inositol production and is the first and rate-limiting one in the synthesis of all inositol-containing compounds. We show that a specific MIPS gene (*PvMIPSs*) is specifically expressed during seed development and its highest levels of expression are detected at very early stages (from 4 to 12 DAF). This very early MIPS expression in seed development has been reported also in other species like rice (Yoshida et al. 1999) and soybean (Hegeman et al. 2001; Chiera and Grabau 2007). Chiera and Grabau (2007) studied MIPS immunolocalisation during soybean seed development and showed that at very early stages GmMIPS is expressed in maternal tissues and then in embryo, thus suggesting a key role in supplying inositol for early embryo development. Of course MIPS should also plays a role in phytic acid synthesis and indeed highest levels of *PvMIPSs* expression precede synthesis and accumulation of inositol phosphates (InsP_3 to InsP_5) which synthesis begins around 12 DAF and peaks at 21 DAF (Coehlo et al. 2005).

We found that also *PvIMP* and *PvIPK2* expression is regulated during seed development, however, they have different timing of expression: while *PvMIPSs* is expressed at very early stages (4–12 DAF), *PvIMP* expression declines slightly later (14–16 DAF) and finally *PvIPK2* expression peaks between 12–14 DAF and slowly decreases during the later developmental stages. This type of regulation suggests a different and sequential time of action for the enzymatic activities coded by these genes and is in agreement with the timing of synthesis and accumulation of phytic acid and Ins*P*s (InsP_3 to InsP_5) reported by Coehlo et al. (2005) for a number of bean varieties.

In rice, *OsMIPS* and *OsIPK1* genes are developmentally regulated and their expression is coordinated (Suzuki et al. 2007). The other two genes analysed, *OsITP5/6k-4* and *OsITP5/6k-6* (belonging to the ITPKα and ITPKγ subgroups, respectively), show opposite trends for regulation: *OsITP5/6k-4* expression increases after the very early stages of seed development and then remains quite constant, while that of *OsITP5/6k-6* decreases. However, the

authors also showed that these two genes are expressed in different seed tissues, aleurone and embryo, respectively, and suggested that the timing of expression reflects the timing of phytate accumulation in these tissues. Although we could not isolate an *ITPKγ* gene in bean and such a type of gene has not been reported in soybean (Stiles et al. 2008), a species very closely related to common bean, we cannot exclude that such type of gene could be present in the bean genome.

In any case, the expression of both *PvITPKα* and *PvITPKβ* does not resemble that observed for these two genes in rice, while it closely resembles that described by Stiles et al. (2008) for soybean *ITPKα* and *ITPKβ* type genes and by Josefsen et al. (2007) for a rice and barley *ITPKα* gene which is constitutively expressed in all tissues examined as well as during seed development.

Our findings also show that, although two copies of MIPS gene are present in common bean, they have tissue specific pattern of expression and are located in different genomic regions, being mapped on chromosomes Pv1 (*PvMIPSs*) and Pv2 (*PvMIPSv*). In addition, we provide evidence that all the other genes of the phytic acid biosynthetic pathway are single copy genes, a characteristic that minimises the risk of functional redundancy that could mask or reduce the effect of the mutation on phytic acid accumulation in seeds. This gene copy number is confirmed by the comparative mapping analysis: in fact, for each bean gene we never found more than two soybean hortologous. The comparative mapping analysis also allowed the finding of bean molecular markers closely associated to the hortologous soybean genes: distances were ranging from about 1 Kbp (between g2435 and the soybean *IMP* gene) up to 600–1,000 Kbp (between g686–g2371 and the soybean *ITPKα* gene). These markers may represent a useful tool for bean breeders, for example in QTL analysis of important agronomic traits.

Finally, bean TILLING populations are under development and will be soon ready for mutant identification by the reverse genetic approach (Gepts et al. 2008). We believe that our data will be useful for identification of bean *lpa* mutants using TILLING and/or ECOTILLING.

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