Localization of myo-inositol-1-phosphate synthase to the endosperm in developing seeds of Arabidopsis

Naoto Mitsuhashi1,2,*, Maki Kondo3, Satoru Nakaune4, Miwa Ohnishi1,5, Makoto Hayashi3, Ikuko Hara-Nishimura4, Alan Richardson6, Hidehiro Fukaki1, Mikio Nishimura3 and Tetsuro Mimura1,5,†

1 Department of Biology, Graduate School of Science, Kobe University, Rokkodai 1-1, Nada-ku, Kobe, 657-8501 Japan
2 Japan Society for the Promotion of Science (JSPS), Chiyoda-ku, Tokyo, 102-8471 Japan
3 Department of Cell Biology, National Institute for Basic Biology, Okazaki, 444-8585 Japan
4 Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, 606-8502 Japan
5 Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Chuou-ku, Tokyo, 113-0027 Japan
6 CSIRO Plant Industry, PO Box 1600, Canberra, ACT, 2601, Australia

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Abstract

Expression and localization of myo-inositol-1-phosphate synthase (MIPS) in developing seeds of Arabidopsis thaliana was investigated. MIPS is an essential enzyme for production of inositol and inositol phosphates via its circularization of glucose-6-phosphate as the initial step. myo-inositol-6-phosphate (InsP6 or phytic acid) is the predominant form of phosphorus found in seeds and accumulates as a consequence of MIPS action. Three MIPS genes have been identified in Arabidopsis, all of which were expressed not only in siliques but in both leaves and roots. Immunoelectron microscopy using a MIPS antibody showed that MIPS localizes to the cytosol primarily in the endosperm during seed development and not in the embryo. This is consistent with results obtained using fluorescent microscopy and western blot analysis that showed a similar pattern of localization. However, InsP6, which is the final product of inositol phosphate metabolism, was present mainly in the embryo. This suggests that a complex interaction between the endosperm and embryo occurs during the synthesis and subsequent accumulation of InsP6 in developing seeds of Arabidopsis.

Key words: Inositol, inositol phosphates, inositol-6-phosphates, myo-inositol-1-phosphate synthase, phosphate, phytate, vacuole.

Introduction

myo-Inositol hexakisphosphate (InsP6; phytic acid) is the most abundant form of phosphorus found in plant seeds. InsP6 in seed is bound to minerals (K, Ca, Mg, Zn, and Mn) to form inclusion particles (globoids) which are commonly located in protein storage vacuoles (Lott et al., 1979). In germinating seeds, InsP6 is hydrolysed into myo-inositol and inorganic phosphates (Pi) by phytase (myo-inositol hexakisphosphate phosphatase) and used for subsequent seedling growth (Loewus and Murthy, 2000; Raboy, 2003). In yeast, InsP6 is also suggested to be involved in mRNA export from the nucleus (Miller et al., 2004) and chromatin remodelling (Shen et al., 2003). More recently, InsP6 has been found to act as a co-factor for auxin binding to the TIR1 plant hormone auxin receptor (Tan et al., 2007). Thus, InsP6 is attracting increased attention not only as a seed storage component but also as a regulator of cellular functions.

From a nutritional perspective, high InsP6 in grain is considered to be undesirable due to its binding of essential...
micronutrient metals. Considerable effort has therefore been undertaken to isolate low InsP6 mutants from various plant species, including barley (Hatzack et al., 2000; Dorsch et al., 2003), maize (Raboy et al., 2000), rice (Larson et al., 2000; Kuwano et al., 2006), and soybean (Wilcox et al., 2000). One such mutant in soybean showed 50% reduction of InsP6 and an ~10-fold increase in the Pi content of seeds, which was associated with lower expression of a MIPS [Ins(3)P] synthase gene (Hitz et al., 2002). Similarly, RNA interference (RNAi)-mediated silencing of MIPS gene expression in transgenic soybean resulted in a 95% reduction of InsP6 in seeds (Nunes et al., 2006). However, the transgenic lines showed impaired seed development, and antisense lines of MIPS genes in potato plants similarly have altered plant morphology (Keller et al., 1998). These may be caused by important roles in the cellular functions of InsP6.

MIPS mediates the conversion of Glc-6-P to Ins(3)P1, and its activity is essential for the subsequent synthesis of InsP6. myo-inositol can also be derived from Ins(3)P1 via dephosphorylation by myo-inositol-1-phosphate phosphatase (Loewus and Murthy, 2000). Inositol is known to play important roles in plant development. Various derivatives of myo-inositol (e.g. muco-inositol, pinitol) also function in environmental stress tolerance. MIPS is therefore an important enzyme for inositol and inositol phosphate metabolism in plants.

InsP6 synthesis requires either the sequential phosphorylation of Ins(3)P1 or the phosphorylation of phosphatidyl inositol intermediates via myo-inositol. A low phytic acid mutant in maize (lpa3) has a mutation in the gene encoding a myo-inositol kinase (MIK) that is normally expressed in developing embryos (Shi et al., 2005). Another maize low phytic acid mutant (lpa2) has altered expression of an Ins(1,3,4)P3/6 kinase (PPK) gene (Shi et al., 2003), both of which caused a significant reduction in the InsP6 content in seeds with a concomitant increase in Pi. Ins(1,4,5)P3 kinase (AtIPK2) and Ins 2-kinase (AtIPK1) have similarly both been shown to be responsible for InsP6 synthesis in Arabidopsis (Stevenson-Paulik et al., 2005). These results indicate that InsP6 synthesis is complex, involving at least two alternative pathways. As part of this complexity, it is likely that enzyme reactions, such as MIPS action and sequential phosphorylation, are separated in different cells or tissues. In addition, it has been shown that addition of an excess of Pi to Catharanthus roseus suspension-cultured cells induced a large accumulation of InsP6 in the vacuole (Mitsuhashi et al., 2005). Although excess Pi increased InsP6 synthesis, the MIPS and Ins(1,4,5)P3 kinase immuno-contents did not change, suggesting complex regulation of InsP6 synthesis, by not only enzymes but also substrate levels.

In the present study, the focus was on the role of MIPS in InsP6 synthesis in developing seeds of Arabidopsis. Yoshida et al. (1999) have shown that expression of a rice MIPS gene (RINO1) was detected between 4 d and 7 d after anthesis in the scutellum and aleurone layer, coinciding with the appearance of InsP6. Treatment of rice cultured cells with abscisic acid (ABA) and sucrose together also resulted in higher levels of MIPS transcript accumulation, suggesting a synergistic induction of the MIPS gene in developing rice seeds (Yoshida et al., 2002). However, the subcellular localization of MIPS was not reported. More recently, Chiera and Grabau (2007) have shown in soybean seed that MIPS (Gm MIPS1) is localized in non-embryo tissues and appeared to be associated subcellularly with plastid-like structures that stained with MIPS antibody. MIPS activity has been reported to be widely distributed in intracellular compartments including membrane-bound organelles and cell walls, as well as cytoplasm (Lackey et al., 2003). Despite this, the site(s) of InsP6 synthesis in seed cells is poorly understood. Most InsP6 is accumulated in the vacuole, which becomes globoid in seeds, but it is not known how InsP6 is transported to the vacuole. Using electron microscopy, Otegui et al. (2002) observed that in developing seeds of Arabidopsis, globoids with Mg2+, K+, and Ca2+ were present within the embryo and that the endosperm of chalazal cells transiently contain Mn- and/or Zn-phytate. More recently, Shi et al. (2007) reported that the maize lpa1 gene encodes an MRP-type ABC transporter and, since it is known that the maize LPA1 mutant has less InsP6, this ABC transporter may be associated with InsP6 storage.

To clarify InsP6 synthesis during Arabidopsis seed maturation, MIPS localization was examined using immunohistochemistry, and it is shown here that MIPS proteins localize to the cytosol of the Arabidopsis seed endosperm. Moreover, the presence of InsP6 within both embryo and endosperm tissues suggests an interaction between the tissues in the synthesis and subsequent storage of InsP6 in developing seeds of Arabidopsis.

Materials and methods

Plant materials

Seeds of Arabidopsis thaliana (Columbia accession) were surface sterilized with 95% ethanol and then sown onto 0.2% gellan gum (Wako, Tokyo, Japan) in 1/2 MS medium (Wako) with 3 mg l−1 thiamine-HCl, 0.5 mg l−1 pyridoxine, and 5 mg l−1 nicotinic acid. After incubation at 4 °C for 4 d to break dormancy, the seeds were germinated and grown at 23 °C under continuous light. After −14 d the seedlings were transferred into vermiculite medium for subsequent growth.

Developing seeds were harvested from Arabidopsis plants having 10–12 siliques. Seeds harvested from the sixth to eighth siliques were separated into seed coat and embryo using tweezers under a binocular (SZX16, Olympus). The seed coat and embryo were washed with RNase-free water three times to remove fragile endosperm tissues.
RT-PCR and real-time RT-PCR

Total RNA was extracted from tissue using the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) according to protocols provided by the manufacturer. First-strand cDNA was generated by reverse transcription with reverse transcriptase XL AMV (Takara Bio Inc., Shiga, Japan) using oligo(dT) primer, 5'-CTGACTCAGATGTTACCGGATCC-3' and 5'-TCTGATGAGTGTTACCGGATCC-3'. Real-time PCR amplification was performed using the SYBR® Premix Ex TaqTM (Takara Bio Inc.) and a real-time PCR detector (TaqKaRa Smart Cycler II system). PCR was performed using gene-specific oligonucleotide primer pairs based on unique sequences for each pairs MIPS gene and an Act-2 (control) gene. The primer sequences used were: for AtMIPS1 (At4g39800), 5'-GCGGAATTCTGACATGATGTT-3' and 5'-GGCGGATCCATGATGTTGTTGTTT-3'; for AtMIPS2 (At5g10170), 5'-GGGATCCATGATGTTGTTGTTT-3' and 5'-GGCGTAATGTTGTTGTTGTTT-3'; for Actin-2 (Atg18780), 5'-TTGGTTCCAGCCCTCGTTTGT-3' and 5'-ATGCTGCTTGGTGCAAGT-3'. In both PCR methods, the same primers sets were used for each gene.

Preparation of antibodies against MIPS

MIPS antibody was prepared according to Mitsuhashi et al. (2005). An expressed sequence tag (EST) clone (accession no. AV525103) for the Arabidopsis MIPS-2 gene (At4g39800) was provided by Kazusa DNA Research Institute. Oligonucleotide primers 5’-GAAA-TTCAAGTATTGAGGCTCAATGTTGTT-3’ and 5’-CTCGAGCGTGAACCATGATCTTTGTTT-3’ were designed on the basis of N- and C-terminal sequences of the MIPS-2 gene, respectively. The amplified DNA was digested with Xhol and EcoRI, and inserted into the Xhol and EcoRI sites of the pET21a vector (EMD Biosciences, San Diego, CA, USA). The pET21-MIPS2 plasmid was introduced into Escherichia coli strain BL21(DE3) (EMD Biosciences). The recombinant protein was purified via a 6 x His tag by using a HiTrap Chelating HP Column (Amersham Biosciences, Piscatway, NJ, USA) and used as antigen. Specific antisera raised by using gene-specific oligonucleotide primer pairs based on unique sequences for each MIPS gene and an Actin-2 (control) gene. The amplified DNA was digested with Xhol and EcoRI, and inserted into the Xhol and EcoRI sites of the pET21a vector (EMD Biosciences, San Diego, CA, USA). The pET21-MIPS2 plasmid was introduced into Escherichia coli strain BL21(DE3) (EMD Biosciences). The recombinant protein was purified via a 6 x His tag by using a HiTrap Chelating HP Column (Amersham Biosciences, Piscatway, NJ, USA) and used as antigen. Specific antisera raised in rabbit were provided by Shibayagi Co., Ltd (Gunma, Japan).

Preparation of thin sections

Developing Arabidopsis seeds with torpedo-shaped embryos were vacuum infiltrated for 1 h with a fixative that consisted of 4% paraformaldehyde, 1% glutaraldehyde, and 0.06 M sucrose in ultrapure water. Developing seeds were polymerized under a UV lamp and sectioned with a Reichert ultramicrotome (Leica, Basingstoke, Hampshire, UK). Blocks were polymerized under a UV lamp and sectioned with a Reichert ultramicrotome (Leica, Basingstoke, Hampshire, UK). Blocks were polymerized under a UV lamp and sectioned with a Reichert ultramicrotome (Leica, Basingstoke, Hampshire, UK). Blocks were polymerized under a UV lamp and sectioned with a Reichert ultramicrotome (Leica, Basingstoke, Hampshire, UK). Blocks were polymerized under a UV lamp and sectioned with a Reichert ultramicrotome (Leica, Basingstoke, Hampshire, UK).

Immunoelectron microscopy

Immunogold labelling procedures were essentially the same as described previously (Hara-Nishimura et al., 1993), except for the use of anti-AtMIPS2 antibodies (Mitsuhashi et al., 2005). Post-fixation was omitted for immunoelectron microscopy. The samples were dehydrated in a graded dimethylformamide series at –20 °C and embedded in London Resin White (London Resin Co., Basingstoke, Hampshire, UK). Blocks were polymerized under a UV lamp and sectioned with a Reichert ultramicrotome (Leica, Heidelberg, Germany). Ultrathin sections were incubated with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature and then incubated with anti-AtMIPS2 antibodies in blocking solution overnight at 4 °C. The sections were washed with PBS and then incubated for 30 min at room temperature with a solution of protein A-gold (10 nm; GE Healthcare Biosciences, Tokyo, Japan) in the blocking solution. The sections were washed with distilled water and then were stained with uranyl acetate and lead citrate. After staining, all sections were examined with a transmission electron microscope (model 1200EX; JEOL, Tokyo, Japan) at 80 kV.

Immunofluorescence analysis

Developing seeds of Arabidopsis were fixed for 40 min in 7.2% (w/v) formaldehyde, 0.1% (v/v) Nonidet P-40, 10% (v/v) dimethyl-sulphoxide, and 50 mM Na-phosphate buffer, pH 7.2. Seeds were then washed twice with Tris-buffered saline–Tween (TBS-T) for 5 min, incubated in TBS-T containing 5% (w/v) Cellulose Onozuka R-10 (Yakult, Tokyo, Japan) and 2% (w/v) Pectolyase Y-23 (Kikkoman, Tokyo, Japan) for 20 min at 30°C, washed twice with TBS-T, incubated in blocking buffer [2% (w/v) BSA and TBS-T] for 30 min, and then incubated with anti-AtMIPS2 or pre-immune antibodies in the blocking buffer for 40 min. After this the seeds were washed three times for 5 min each, incubated for 1 h with goat anti-rabbit IgG antibodies conjugated with Alexa Fluor 488 (absorbance, 495 nm; emission, 519 nm; Molecular Probes, Eugene, OR), washed three times for 5 min with TBS-T, and mounted.

Immunoblot analysis

Seed tissues of Arabidopsis were homogenized in 10 mM Tris-HCl, pH 7.5, before centrifugation to collect soluble proteins. Proteins (5 μg per lane) were subjected to SDS–PAGE and were transferred electrophoretically to a polyvinylidene difluoride membrane according to the manufacturer’s protocol (Biocraft, Tokyo, Japan). The membrane was incubated with the antibodies against AtMIPS2. Horseradish peroxidase-conjugated donkey antibodies directed against rabbit IgG (GE Healthcare Biosciences) were used as the secondary antibodies. The antibody-labelled proteins were visualized with an enhanced chemiluminescence kit (ECL system; GE Healthcare Biosciences). Antibodies against Arabidopsis 2S albumin and 12S globulin were also used as controls (Shimada et al., 2003).

Measurement of inositol phosphates

Inositol phosphates and Pi were measured using a DX-500 ion chromatography system (Dionex, Osaka, Japan) consisting of a gradient pump, a 25 μl sample loop, and a conductivity detector as described previously (Baluyot and Hartford, 1996; Sekiguchi et al., 2005; Mitsuhashi et al., 2005). Dionex IonPac AS11 (2 mm ID×250 mm) and IonPac AG11 (2 mm ID×50 mm) columns packed with anion-exchange resin were used as the separation columns. The Dionex ASRS-Ultra anion self-regenerating suppressor was operated in the external water mode at 100 mA. The Dionex PeakNet workstation was used for data processing. A Dionex EG40 eluent generator equipped with an EGC-KOH cartridge was used. A Dionex IonPac ATC-1 (4 mm ID×35 mm), a high-capacity anion-exchange column, was placed at the pump outlet to remove the small amount of dissolved carbon dioxide and carbonate in the deionized water. This procedure allowed InsP3 to InsP6 to be resolved in a single gradient elution. A 25 μl aliquot of the filtered samples was automatically injected by an autosampler AS-50 (Dionex). The flow-rate was 0.35 ml min⁻¹ at 35 °C. The concentration gradient (5–80 mM KOH) was generated by EG40. The detection limit (S/N = 3) for InsP2 was 50 nM. Mitsuhashi et al. (2005) showed chromatograms of all 64 isomers of Inps. They finally separated three of six InsP2 isomers, three of 15 InsP3 isomers, eight of 20 InsP4 isomers, eight of 15 InsP5 isomers, and three of six InsP6 isomers independently, although enantiomers were not separated. InsP2 was purchased from Sigma (St Louis, MO, USA).
Results

Investigation of MIPS gene expression in Arabidopsis

The objective of the present work is to clarify the localization of InsP6 synthesis during seed maturation in Arabidopsis. Conversion of Glc-6-P to Ins(3)P1 is mediated by MIPS which is an initial key step in InsP6 synthesis. In the Arabidopsis genome, there are three isoforms of the MIPS gene, i.e. AtMIPS1 (At2g22240), AtMIPS2 (At4g39800), and AtMIPS3 (At5g10170). To elucidate whether any one of these genes is responsible for InsP6 synthesis in developing seeds, the expression pattern of the MIPS isoforms was investigated using RT-PCR. As the nucleotide sequences of the three genes is highly conserved (>79%), specific PCR primers were designed for the C-terminus-encoding and 3’-untranslated region (UTR) sequences, respectively. All three AtMIPS genes were expressed in developing siliques, leaves, and roots (Fig. 1a), suggesting that the AtMIPS1, AtMIPS2, and AtMIPS3 genes may also all be expressed in developing seeds. Real-time PCR analysis using the same primer sets revealed that the relative expression of AtMIPS1 and AtMIPS2 in developing siliques was higher than that of AtMIPS3.

Immunolocalization of MIPS proteins in developing seeds of Arabidopsis

In Arabidopsis, it is not clear when InsP6 accumulation is initiated during seed development. Two different stages (torpedo and mature) of developing seeds were therefore investigated for localization of AtMIPS proteins with immunoelectron microscopy using a polyclonal antibody raised against AtMIPS2. However, this antibody does not distinguish between the three isoforms of MIPS due to high amino acid sequence homology (>89%) across the proteins. Indeed, MIPS proteins of another species (Catharanthus roseus) were also detected with this antibody (Mitsuhashi et al., 2005). The immunolocalization data obtained therefore are likely to represent the additive signals of the three AtMIPS proteins which were all expressed in developing siliques. The density of immunogold particles showed a similar frequency between cells in torpedo and mature stages (Fig. 2). The subcellular

![Fig. 1. Expression of MIPS genes in Arabidopsis. (a) Organ specificity of MIPS gene expression. Shown is RT-PCR of total RNA extracted from green siliques (Silique), mature leaves (Leaf), and roots (Root) using gene-specific primers for the Arabidopsis AtMIPS1 (At2g22240), AtMIPS2 (At4g39800), AtMIPS3 (At5g10170), and ACTIN2 (ACT2; At3g18780) genes. (b) Relative expression levels of the three MIPS genes relative to the ACT2 gene in green siliques measured with real-time PCR. Values are expressed with mean ± SE (n = 3).](image)

![Fig. 2. Immunelectron microscopy of MIPS protein in Arabidopsis seeds. (A) Transverse section of whole mature seeds. (B–D) Thin sections of torpedo-stage seeds (B and C) and mature-stage seeds (D and E). B and D show embryo tissue, and C and E show endosperm. CW, cell wall; Mt, mitochondria. Arrowheads show gold particles with anti-AtMIPS2 antibody.](image)
Localization indicated the presence of immunogold particles mainly in the cytosol of the endosperm (Fig. 2C, E; arrowheads), with negligible presence in other organelles, cell walls, and membrane structures. Both torpedo and mature stage embryonic cells showed only low background levels of gold particles (Fig. 2), indicating that AtMIPS proteins appear to be specifically located within the endosperm cytosol.

Localization of AtMIPS proteins was also investigated using immunofluorescent photomicroscopy. Mature stage seed sections were exposed to anti-AtMIPS2 serum or pre-immune control serum, and then to a secondary antibody with a fluorescent conjugate (Alexa Fluor 488). Consistent with the immunolocalization results, the fluorescent signal of the anti-AtMIPS antibody was also detected in the endosperm only and not in the embryo (Fig. 3). Control serum showed no positive signals in any cells.

In addition to immunohistochemical analysis, the tissue-specific location of MIPS was also investigated by western blot analysis. Developing seeds were collected from Arabidopsis plants with between 10 and 12 siliques. Individual seeds were then dissected to separate the seed contents, containing the embryo and its surrounding endosperm, from the seed coat. In some cases, the endosperm was also removed from the embryo by washing with Tris buffer, and protein distributions were then assayed using MIPS, 2S albumin, and 12S globulin antibodies. AtMIPS protein (60 kDa band) showed low abundance in the seed coat and was detected primarily in non-washed tissues only, with a marked reduction when the endosperm was removed (Fig. 4). In contrast, the 2S albumin and 12S globulin seed storage proteins showed high abundance in both unwashed and washed tissues, suggesting either an embryo-specific location or a presence in both embryo and endosperm tissues.

Localization of Ins$P_6$ in Arabidopsis seeds

The presence of Ins$P_6$ was similarly measured in embryo and embryo+endosperm tissues using ion chromatography. Ins$P_6$ was clearly detected in the embryo and was not reduced by removal of the endosperm by washing (Fig. 5 and Supplementary Fig. S1 available at JXB online). This suggests that Ins$P_6$ accumulates primarily within the embryo despite the AtMIPS proteins being predominantly localized to the endosperm, as determined by the immunohistochemical analyses. Therefore, it is evident that the site of initial synthesis of inositol phosphates in Arabidopsis seeds (through the action of MIPS genes) may differ from the site of eventual accumulation of Ins$P_6$ and that there is interaction between the endosperm and embryo in the transport of Ins$P_6$ (or lower order derivatives) across the different tissues.

Discussion

Arabidopsis contains three isoforms of the MIPS gene which were ubiquitously expressed in the various organs examined.

Fig. 3. Immunofluorescence microscopy of MIPS protein in mature seed sections. Sections were treated with either anti-AtMIPS2 serum (left) or pre-immune serum (right) followed by a secondary anti-rabbit antibody with a fluorescent conjugate (Alexa Fluor 488). DIC, differential interference contrast images; FL, fluorescence images. The arrowhead shows embryo and the arrow shows endosperm tissue.
(i.e. developing siliques, leaves, and roots). MIPS proteins may be involved not only in InsP₆ synthesis in developing seeds, but also in other physiological processes, such as raffinose metabolism (Hitz et al., 2002), salt tolerance (Ishitani et al., 1996; RayChaudhuri and Majumder, 1996), and ABA responses (Flores and Smart, 2000; Yoshida et al., 2002), via inositol or phosphatidylinositol synthesis. In Arabidopsis, AtMIPS gene isoforms may similarly have broad functionality in different tissues. However, differences of tissue specificity and the isoform(s) that is responsible for InsP₆ synthesis in developing seeds remain unclear.

Although it was evident that all three isoforms were expressed in developing siliques (Fig. 1), InsP₆ synthesis requires many enzymatic steps after MIPS reaction. It is reasonable to suppose that the complex pathways for InsP₆ synthesis exist separately in different tissues or different cellular compartments. In the present study, it was found that MIPS proteins localize in endosperm, but InsP₆ was mainly accumulated in the embryo. The relationship between MIPS reaction and InsP₆ synthesis is not direct. However, it is also known that MIPS mutation decreases InsP₆ accumulation (Hitz et al., 2002). Thus, identification of MIPS localization would help to clarify the mechanism of InsP₆ synthesis during seed maturation.

**Localization of AtMIPS proteins in developing seeds**

Immunoelectron microscopy and immunofluorescent confocal microscopy of developing seeds revealed that AtMIPS proteins in Arabidopsis were predominantly localized to the cytosol within the seed endosperm. The presence of MIPS within the endosperm was further verified by immunolocalization on western blots. In Figs 2–4, a small amount of MIPS proteins was also detectable in the embryo. Although it cannot be concluded that this resulted from contamination, the largest amounts of MIPS exist in the endosperm. Unfortunately, the antibody used could not distinguish between the three MIPS isoforms, so it was not possible to determine the relative contribution of each of the proteins to the localization that was observed, and thus the specific function of the different isoforms cannot yet be elucidated. Also, the antibody could not detect post-translational modifications, such as phosphorylation. Thus, the present data may not be related to the real activity of MIPS proteins. It is important to measure the enzymatic activity of MIPS in the embryo and endosperm. This is a possible subject of future research.

In monocots, InsP₆ has been shown to accumulate in both the embryo and aleurone layer, whereas in dicots it accumulates only within the cotyledons of the embryo (Reddy and Sathe, 2002). In rice, InsP₆-containing globoids have been shown to appear within the scutellum and aleurone layers of developing seeds, which coincides with the localization of the RINO1 (a rice MIPS gene) transcript (Yoshida et al., 1999). In contrast, for Arabidopsis it is shown that AtMIPS proteins localized to the endosperm, whilst InsP₆ accumulated predominantly within the embryo. This raises an interesting question as to why the localization of MIPS proteins does not appear to coincide with that of accumulation of InsP₆, and suggests some degree of cooperativity between the two tissues.

Conversion from Glc-6-P to Ins(3)P₁ is considered to be one of the rate-limiting steps for InsP₆ synthesis (Hitz et al., 2002; Kuwano et al., 2006; Nunes et al., 2006). Based on the present observations, Arabidopsis embryos did not contain a significant amount of MIPS relative to endosperm, and therefore are unlikely to be the site for
initial synthesis of InsP₆. It is more likely that synthesis of InsP₆ is dependent on precursors provided by the endosperm, which is consistent with that recently reported for soybean (Chiera and Grabau, 2007). In one of the pathways of InsP₆ synthesis, Ins(3)P₁ synthesized by MIPS activity is first broken down to Ins and then rephosphorylated to Ins(3)P₁. This represents a futile cycle if it occurs within one cell. Perhaps these steps are separated in different cells or tissues. However, further work to establish tissue-dependent metabolism of InsP₆ metabolism in developing seeds is required.

Translocation from endosperm to embryo

During seed development, InsP₆ and minerals (Mn and Zn) have been shown to be transiently stored in charazal endosperm of Arabidopsis (Otegui et al., 2002) or small particles of castor bean (Greenwood and Bewley, 1984). In Arabidopsis seed, the endosperm serves to support the growth and development of the embryo. As for most dicot seeds (including Arabidopsis) the endosperm is ephemeral, and stored nutrients are absorbed by the embryo as the seed matures (Berger, 1999, 2003). However, as symplasmic connections are not present between the endosperm and embryo (Patrick and Offer, 2001), nutrients must be transferred through membrane transporters or via specialized cells, such as the transfer cells of the cotyledons or other contributing parts (Thompson et al., 2001). If the initial step of MIPS-mediated synthesis of InsP₆ occurs in the endosperm, and InsP₆ subsequently accumulates in the embryo, then there is a need for transport of InsP₆ or its precursors to the embryo. Whilst the presence of such transporters for transfer of phosphorylated inositols has not been reported, a recent study by Shi et al. (2007) has identified a role for an ABC transporter in InsP₆ accumulation in corn and it may function in the transport of phosphorylated inositols. Alternatively, it is possible that Ins(3)P₁ is dephosphorylated by inositol monophosphatase within the endosperm and transported as myo-inositol to the embryo via an inositol transporter. Translocated myo-inositol may then be phosphorylated sequentially to InsP₆ by inositol kinases. In preliminary experiments, the expression of both inositol transporter genes (four isoforms) and several InsP₃ kinase genes has been detected in siliques from Arabidopsis (Supplementary Fig. S2 at JXB online). An EST clone (accession no. AV525103) was kindly provided by Kazusa DNA Research Institute. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (16085204) by the Japanese Ministry of Education, Culture, Sports, Science and Technology, and CREST of JST to TM. NM was supported by a JSPS research fellowship for young scientists.

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Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Examples of a chromatogram of InsP₆ measurements with ion chromatography. (a) InsP₆ in endosperm and embryo. (b) InsP₆ in embryo after washing with pure water. (c) InsP₆ in seed coat.

Fig. S2. Expression of inositol transporters (a) and several InsP₃ kinase genes (b) in silique, leaf, and root tissue. PPK, inositol 1,3,4-trisphosphate 5/6-kinase; ITR, inositol transporter; IPK2, Ins₁,4,5-P₃ kinase. The following primer sets were used for RT-PCR, AtITR1 (At4g16480) 5’-GCATGCTTTATCTTCTGACCGACAG-3’ and 5’-GCCCCAAAACACTATAGCTAAGA-3’, AtITR2 (At2g35740) 5’-ACTTGTGCTTGTACATTTGCT-3’ and 5’-AAGGAGAACATATGGCCAGGT-3’, AtITR3 (At2g43330) 5’-GATAGTCAAATTGGCTGTGGTT-3’ and 5’-CTAAGGCAAGCAGCGAG-3’, AtITR4 (At1g30220) 5’-CATTATCTGCAGAAATACTAACCG-3’ and 5’-GTCCCAATCAGAAGAGCA-3’, PPK1 (At5g16760) 5’-TCGAAACACTCAAGGCAACGA-3’ and 5’-TCCGGGA-CACCAATCTCCT-3’, PPK2 (At4g08170) 5’-CCGGAGGCCTGTCAATATGC-3’ and 5’-CCGGAGGCCTGTCAATATGC-3’, PPK3 (At4g33770) 5’-GCAGACCTGGACCTCTGAGTGTG-3’ and 5’-TTTAGGGAATTGATGATGCTGATACTAAGA-3’, PPK2 (At5g07370) 5’-AAGGGAATTGATGATGCTGATACTAAGA-3’, PPK2 (At5g61760) 5’-TCCGGGA-CACCAATCTCCT-3’ and 5’-CTAAGGCAAGCAGCGAG-3’.

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