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

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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 (InsP$_6$ 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, InsP$_6$, 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 InsP$_6$ in developing seeds of *Arabidopsis*.

Introduction

myo-Inositol hexakisphosphate (InsP$_6$; phytic acid) is the most abundant form of phosphorus found in plant seeds. InsP$_6$ 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, InsP$_6$ 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, InsP$_6$ 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, InsP$_6$ has been found to act as a co-factor for auxin binding to the TIR1 plant hormone auxin receptor (Tan *et al.*, 2007). Thus, InsP$_6$ is attracting increased attention not only as a seed storage component but also as a regulator of cellular functions.

From a nutritional perspective, high InsP$_6$ in grain is considered to be undesirable due to its binding of essential

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micronutrient metals. Considerable effort has therefore been undertaken to isolate low $InsP_6$ 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 $InsP_6$ and an ~10-fold increase in the Pi content of seeds, which was associated with lower expression of a *MIPS* [$Ins(3)P_1$ 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 $InsP_6$ 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 $InsP_6$.

MIPS mediates the conversion of Glc-6-P to $Ins(3)P_1$, and its activity is essential for the subsequent synthesis of $InsP_6$. *myo*-inositol can also be derived from $Ins(3)P_1$ 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.

$InsP_6$ synthesis requires either the sequential phosphorylation of $Ins(3)P_1$ 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 $Ins(1,3,4)P_3$ 5/6 kinase (PPK) gene (Shi *et al*., 2003), both of which caused a significant reduction in the $InsP_6$ content in seeds with a concomitant increase in Pi. $Ins(1,4,5)P_3$ kinase (*AtIPK2*) and Ins 2-kinase (*AtIPK1*) have similarly both been shown to be responsible for $InsP_6$ synthesis in *Arabidopsis* (Stevenson-Paulik *et al*., 2005). These results indicate that $InsP_6$ 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 $InsP_6$ in the vacuole (Mitsuhashi *et al*., 2005). Although excess Pi increased $InsP_6$ synthesis, the MIPS and $Ins(1,4,5)P_3$ kinase immuno-contents did not change, suggesting complex regulation of $InsP_6$ synthesis, by not only enzymes but also substrate levels.

In the present study, the focus was on the role of MIPS in $InsP_6$ 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, co-inciding with the appearance of $InsP_6$. 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 $InsP_6$ synthesis in seed cells is poorly understood. Most $InsP_6$ is accumulated in the vacuole, which becomes globoid in seeds, but it is not known how $InsP_6$ is transported to the vacuole. Using electron microscopy, Otegui *et al*. (2002) observed that in developing seeds of *Arabidopsis*, globoids with Mg^{2+}, K^+, and Ca^{2+} 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 $InsP_6$, this ABC transporter may be associated with $InsP_6$ storage.

To clarify $InsP_6$ synthesis during *Arabidopsis* seed maturation, MIPS localization was examined using immunohistology, and it is shown here that MIPS proteins localize to the cytosol of the *Arabidopsis* seed endosperm. Moreover, the presence of $InsP_6$ within both embryo and endosperm tissues suggests an interaction between the tissues in the synthesis and subsequent storage of $InsP_6$ 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′-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTTTTTTTTT. Real-time PCR amplification was performed using the SYBR® Premix Ex Taq™ (TaKaRa Bio Inc.,) and a real-time PCR detector (TaKaRa Smart Cycler II system). PCR was performed using gene-specific oligonucleotide primer pairs based on unique sequences for each *MIPS* gene and an *Actin*-2 (control) gene. The primer sequences used were: for *AtMIPS1* (At2g22240), 5′-GCGGGATCCCATGGAGTACAAGTGAAGGATGAG-3′ and 5′-GCGGAATTCGAAAATCCATATTCATAGATCATAAG-3′; *AtMIPS2* (At4g39800), 5′-GCGGAATTCAAGTGAACATGA-AGAAGCATGAAC-3′ and 5′-GCGATCGATGGAACCAAA-ACCATGATTATATATCTC-3′; *AtMIPS3* (At5g10170), 5′-GCGATCGATTCTCGAGTACAAGTGATCAAAGAGAC-3′ and 5′-GCGCTCGAGCCCAAATATATATTATAGTTTGAAATG-3′; and for *Actin*-2 (At3g18780), 5′-TTTGTTCCAGCCCTCGTTTGT-3′ and 5′-TCATGCTGCTTGGTGCAAGT-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′-GAA-TTCATGTTTATTGAGAGCTTCAAAGTT-3′ and 5′-CTCGAGC-TTGAACTCCATGATCATGTTGTT-3′ were designed on the basis of N- and C-terminal sequences of the *MIPS-2* gene, respectively. The amplified DNA was digested with *Xho*I and *Eco*RI, and inserted into the *Xho*I and *Eco*RI 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×His tag by using a HiTrap Chelating HP Column (Amersham Biosciences, Piscataway, 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 0.05 M cacodylate buffer, pH 7.4. The tissues were cut into slices of <1 mm in thickness with a razor blade and treated for another 2 h with freshly prepared fixative.

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) Cellulase 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.*, 2000; 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 $InsP_1$ to $InsP_6$ 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 $InsP_6$ was 50 nM. Mitsuhashi *et al.* (2005) showed chromatograms of all 64 isomers of $InsP_n$. They finally separated three of six $InsP_1$ isomers, three of 15 $InsP_2$ isomers, eight of 20 $InsP_3$ isomers, eight of 15 $InsP_4$ isomers, and three of six $InsP_5$ isomers independently, although enantiomers were not separated. $InsP_6$ 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 InsP$_6$ synthesis during seed maturation in *Arabidopsis*. Conversion of Glc-6-P to Ins(3)P$_1$ is mediated by MIPS which is an initial key step in InsP$_6$ 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 InsP$_6$ 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 sa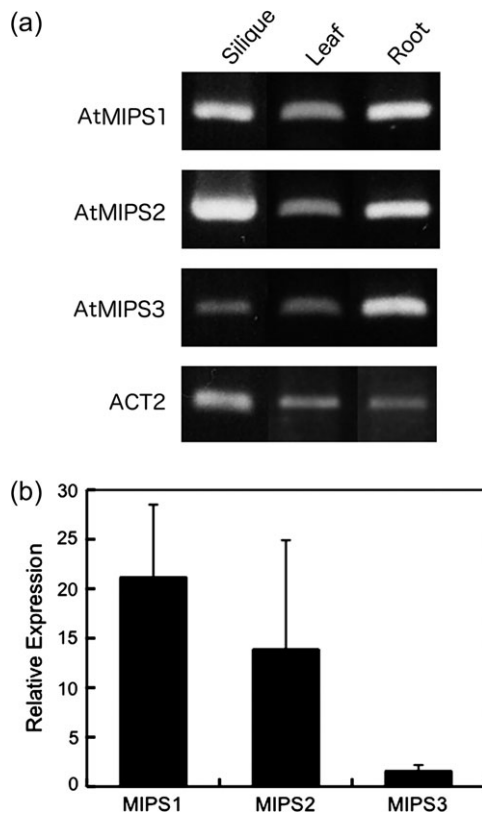me primer sets revealed that the relative expression of *AtMIPS1* and *AtMIPS2* in developing siliques was higher than that of *AtMIPS3*.

Immunolocalization of MIPS proteins in developing seeds of Arabidopsis

In *Arabidopsis*, it is not clear when InsP$_6$ 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
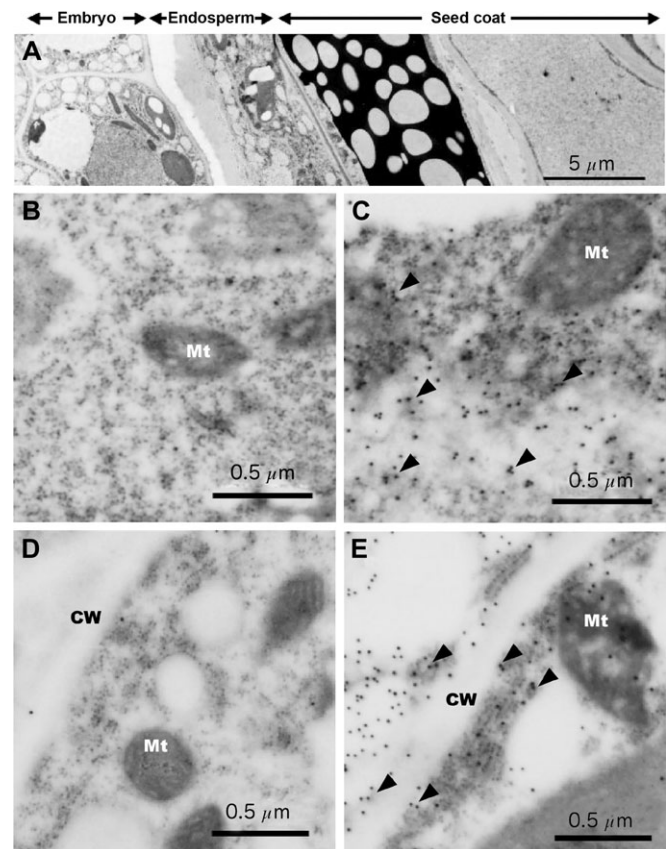

Fig. 1. Expression of *MIPS* genes in *Arabidopsis*. (a) Organ specificity of *MIPS* gene expression. Shown is RT-PCR of total RNA extracted from green siliques (Silique), mature leaves (Leaf), and roots (Root) using gene-specific primers for the *Arabidopsis AtMIPS1* (At2g22240), *AtMIPS2* (At4g39800), *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).

Fig. 2. Immunoelectron 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.

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 InsP$_6$ in Arabidopsis *seeds*

The presence of InsP$_6$ was similarly measured in embryo and embryo+endosperm tissues using ion chromatography. InsP$_6$ 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 InsP$_6$ 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 InsP$_6$ and that there is interaction between the endosperm and embryo in the transport of InsP$_6$ (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
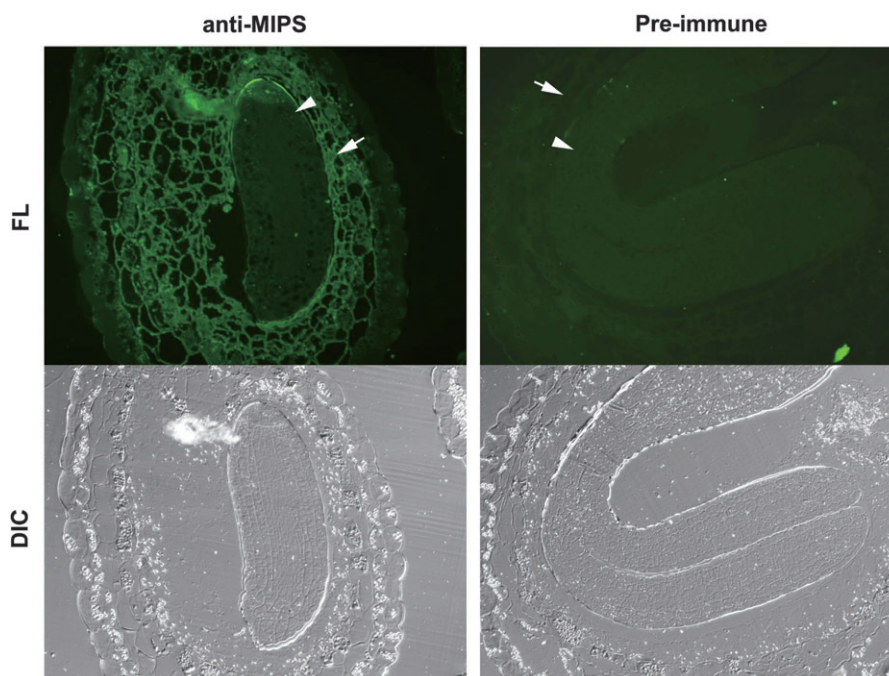

Fig. 3. Immunofluorescence microscopy of MIPS protein in mature seed sections. Sections were treated with either anti-AtMIPS2 serum (left) or pre-immune serum (right) followed by a secondary anti-rabbit antibody with a fluorescent conjugate (Alexa Fluor 488). DIC, differential interference contrast images; FL, fluorescence images. The arrowhead shows embryo and the arrow shows endsperm tissue.
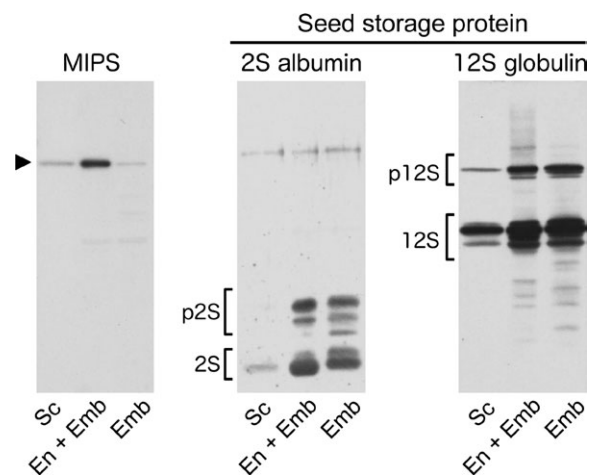
Fig. 4. Tissue specificity of MIPS and seed storage proteins in developing seeds of *Arabidopsis* as determined by immunoblot analysis with MIPS2, 2S albumin, and 12S globulin antibodies. Developing seeds (torpedo to mature stages) were separated into seed coat (Sc) and seed contents which were either left intact as endosperm and embryo (En+Emb), or washed to remove the endosperm (Emb). p2S and 2S are pro- and mature forms of 2S albumin, and p12S and 12S are pro- and mature forms of 12S globulin, respectively. The arrowhead at 60 kDa indicates MIPS.
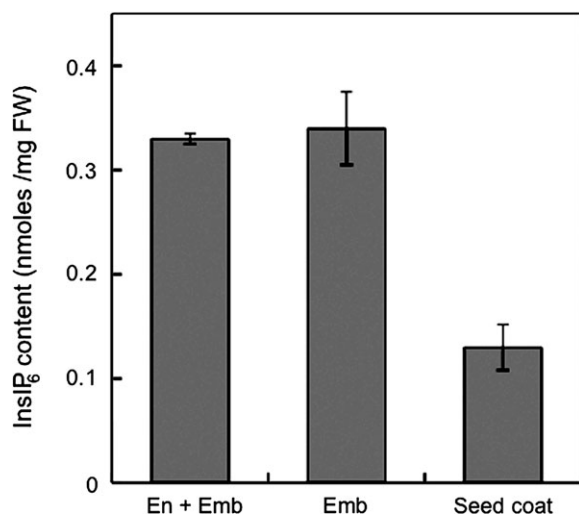

Fig. 5. InsP$_6$ content in endosperm, embryo tissue, and seed coat. InsP$_6$ was measured using ion chromatography. Values are expressed with mean ± SE (n = 3–6).

(i.e. developing siliques, leaves, and roots). MIPS proteins may be involved not only in InsP$_6$ 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 *Arabidopsi*s, *AtMIPS* gene isoforms may similarly have broad functionality in different tissues. However, differences of tissue specificity and the isoform(s) that is responsible for InsP$_6$ synthesis in developing seeds remain unclear.

Although it was evident that all three isoforms were expressed in developing siliques (Fig. 1), InsP$_6$ synthesis requires many enzymatic steps after MIPS reaction. It is reasonable to suppose that the complex pathways for InsP$_n$ synthesis exist separately in different tissues or different cellular compartments. In the present study, it was found that MIPS proteins localize in endosperm, but InsP$_6$ was mainly accumulated in the embryo. The relationship between MIPS reaction and InsP$_6$ synthesis is not direct. However, it is also known that MIPS mutation decreases InsP$_6$ accumulation (Hitz *et al.*, 2002). Thus, identification of MIPS localization would help to clarify the mechanism of InsP$_6$ synthesis during seed maturation.

Localization of AtMIPS proteins in developing seeds

Immunoelectron microscopy and immunofluorescent photomicroscopy 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, InsP$_6$ 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, InsP$_6$-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 InsP$_6$ 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 InsP$_6$, and suggests some degree of cooperativity between the two tissues.

Conversion from Glc-6-P to Ins(3)P$_1$ is considered to be one of the rate-limiting steps for InsP$_6$ 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 $InsP_6$. It is more likely that synthesis of $InsP_6$ 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 $InsP_6$ synthesis, $Ins(3)P_1$ synthesized by MIPS activity is first broken down to Ins and then rephosphorylated to $Ins(3)P_1$. 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 $InsP_n$ metabolism in developing seeds is required.

Translocation from endosperm to embryo

During seed development, $InsP_6$ 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 Offler, 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 $InsP_6$ occurs in the endosperm, and $InsP_6$ subsequently accumulates in the embryo, then there is a need for transport of $InsP_6$ 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 $InsP_6$ accumulation in corn and it may function in the transport of phosphorylated inositols. Alternatively, it is possible that $Ins(3)P_1$ 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 $InsP_6$ by inositol kinases. In preliminary experiments, the expression of both inositol transporter genes (four isoforms) and several $InsP_3$ kinase genes has been detected in siliques from *Arabidopsis* (Supplementary Fig. S2 at *JXB* online). An attempt has also been made to detect the expression differences of MIPS and those genes between the endosperm and embryo, but the expression of all genes in both tissues was always detected, because mRNA contamination could not be removed from each tissue. Clearly, there is need for a better understanding of the role of specific genes in inositol phosphate metabolism in developing seeds, and of the relationship between endosperm and embryo tissues for $InsP_6$ synthesis.

In the present study, it was shown that three isoforms of MIPS are expressed in developing siliques and that the MIPS protein localizes to the cytosol of the seed endosperm. Such an observation has important implications for the synthesis of $InsP_6$, which was found to be present predominantly within the embryo of developing and mature *Arabidopsis* seeds.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Examples of a chromatogram of $InsP_6$ measurements with ion chromatography. (a) $InsP_6$ in endosperm and embryo. (b) $InsP_6$ in embryo after washing out with pure water. (c) $InsP_6$ in seed coat.

Fig. S2. Expression of inositol transporters (a) and several $InsP_3$ kinase genes (b) in silique, leaf, and root tissue. PPK, inositol 1,3,4-trisphosphate 5/6-kinase; ITR, inositol transporter; IPK2, $Ins(1,4,5)P_3$ kinase. The following primer sets were used for RT-PCR, *AtITR1* (At4g16480) 5′-GCATGTCTTATCATCTTAGCCACG-3′, 5′-GCCCC-AAAAACACTATAGCTAAGA-3′, *AtITR2* (At2g35740) 5′-ACTTGCCTTGTCATTTTGGCT-3′ and 5′-AAG-GAAGACAATGGCCAGGT-3′, *AtITR3* (At2g43330) 5′-GATAGTCCAAATGGCTGGATTTC-3′ and 5′-CTA-AGCCAAGCACAGCGAG-3′, *AtITR4* (At1g30220) 5′-CATTATCTCGCTAGGAATACTAACCG-3′ and 5′-GT-CCCAATCCAAGAAGAGCA-3′, *PPK1* (At5g16760) 5′-TCGAACACTCAAGGCAACGA-3′ and 5′-TCCGGGA-CACCAAATCTCTC-3′, *PPK2* (At4g08170) 5′-CCGG-AGGCTGTCAATAATGC-3′ and 5′-CACAAGAGAC-CCGTGGGAAG-3′, *PPK3* (At4g33770) 5′-GCAGA-CTTGGACCCTCGTGT-3′ and 5′-TTTGACCTGCGC-CAGATTTT-3′, *AtIPK2α* (At5g07370) 5′-TAAAGGGA AATGATGATGATGCTA-3′ and 5′-CTAAGAATCTG-CAGACTCATCTGG-3′, *AtIPK2β* (At5g61760)5′-CAA-TCTTGATGCAAGGAGGAG-3′ and 5′-CTAGCGCCC-GTTCTCAAGT-3′.

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