Phytic Acid Synthesis and Vacuolar Accumulation in Suspension-Cultured Cells of *Catharanthus roseus* Induced by High Concentration of Inorganic Phosphate and Cations[1][w]

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We have established a new system for studying phytic acid, *myo*-inositol hexakisphosphate ($InsP_6$) synthesis in suspension-cultured cells of Catharanthus. $InsP_6$ and other intermediates of *myo*-inositol (Ins) phosphate metabolism were measured using an ion chromatography method. The detection limit for $InsP_6$ was less than 50 nM, which was sufficient to analyze Ins phosphates in living cells. Synthesis of Ins phosphates was induced by incubation in high inorganic phosphate medium. $InsP_6$ was mainly accumulated in vacuoles and was enhanced when cells were grown in high concentration of inorganic phosphates with the cations K^+, Ca^{2+}, or Zn^{2+}. However, there was a strong tendency for $InsP_6$ to accumulate in the vacuole in the presence of Ca^{2+} and in nonvacuolar compartments when supplied with Zn^{2+}, possibly due to precipitation of $InsP_6$ with Zn^{2+} in the cytosol. A vesicle transport inhibitor, brefeldin A, stimulated $InsP_6$ accumulation. The amounts of both Ins(3)P_1 *myo*-inositol monophosphate synthase, a key enzyme for $InsP_6$ synthesis, and Ins(1,4,5)P_3 kinase were unrelated to the level of accumulation of $InsP_6$. The mechanisms for $InsP_6$ synthesis and localization into vacuoles in plant cells are discussed.

myo-Inositol (Ins) phosphates play crucial roles in both animal and plant cells. In plants, a large amount of *myo*-inositol hexakisphosphate ($InsP_6$; phytic acid) is synthesized and accumulated in seeds as a phosphorus reservoir instead of inorganic phosphates (Pi). $InsP_6$ also has various physiological roles other than storage of phosphorus, such as mRNA export (Miller et al., 2004) and chromatin remodeling (Shen et al., 2003).

Research into Ins phosphates in plants first concentrated on measuring levels in seeds, mainly of $InsP_6$ (Holt, 1955; Asada et al., 1969; Griffiths and Thomas, 1981; Raboy et al., 1984), and on the investigation of reaction kinetics of the enzymes involved in synthesis of $InsP_6$ (Phillippy et al., 1994; Phillippy, 1998; Brearley and Hanke, 2000; Stevenson-Paulik et al., 2002). From a human nutrition perspective, high $InsP_6$ in grain is considered undesirable due to its binding of essential micronutrient metals. Low-$InsP_6$ mutants have been isolated from barley (*Hordeum vulgare*; Hatzack et al., 2000; Dorsch et al., 2003), maize (*Zea mays*; Raboy et al., 2000), rice (*Oryza sativa*; Larson et al., 2000), and soybean (*Glycine max*; Wilcox et al., 2000). A maize low-phytic acid mutant *lpa2* arose by mutation in an Ins(1,3,4)P_3 5/6 kinase gene (Shi et al., 2003), which caused the $InsP_6$ content to be reduced by approximately 30%, and the Pi to increase about 3-fold. Another

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mutant in maize (*lpa241*) showed approximately 90% reduction of $InsP_6$, 10-fold increase in seed-free phosphate content, and reduction of $Ins(3)P_1$ synthase gene expression (Pilu et al., 2003).

Many aspects of the synthesis of $InsP_6$ are also well investigated. The early step of $InsP_6$ synthesis is the conversion of Glu-6-P to $Ins(3)P_1$ mediated by $Ins(3)P_1$ *myo*-inositol monophosphate synthase (MIPS). The latter steps are consistent with sequential phosphorylations of soluble Ins phosphates mediated by several kinases and phospholipase C-dependent conversion of phosphatidyl inositol phosphate intermediates to $Ins(1,4,5)P_3$ (Loewus and Murthy, 2000; Raboy, 2003). MIPS gene expression is suppressed by addition of Ins (Johnson and Sussex, 1995).

In spite of these efforts, the understanding of the factors that regulate synthesis of $InsP_6$ is patchy. During seed maturation, the accumulation of $InsP_6$ in seeds was strongly affected by the level of Pi in the culture solution (Asada et al., 1969; Raboy and Dickinson, 1984). Yoshida et al. (1999) showed that strong signals of transcript of a rice MIPS gene (RINO1) were detected between 4 and 7 d after anthesis in scutellum and aleurone layer, coinciding with the appearance of phytin. They subsequently reported that treatment of rice cultured cells with abscisic acid and Suc together resulted in much higher levels of MIPS transcript accumulation, suggesting a synergistic induction of the MIPS gene (Yoshida et al., 2002). It was recently shown that MIPS activity is widely distributed in intracellular compartments, including membrane-bound organelles and cell walls, as well as cytoplasm (Lackey et al., 2003), but the site(s) of $InsP_6$ synthesis has not been established, nor is it known how $InsP_6$ is transported to the vacuole, which becomes globoid in seeds. Most of the $InsP_6$ in castor bean (*Ricinus communis*) seeds is bound to Ca^{2+} and Mg^{2+} and accumulated in globoid in protein storage vacuoles (Greenwood and Bewley, 1984). In developing seeds of Arabidopsis (*Arabidopsis thaliana*), embryo globoids contained Mg^{2+}, K^+, and Ca^{2+}, the charazal endoplasmic reticulum (ER) showed high level of Mn^{2+}, and the charazal vacuoles contained zinc phytate (Otegui et al., 2002).

Here, we report the development of an experimental system for the in vivo investigation of the dynamics of synthesis and compartmentation of $InsP_6$ using suspension-cultured cells of Catharanthus.

RESULTS

Induction of $InsP_6$ Accumulation in Suspension-Cultured Cells

Although most interest in $InsP_6$ is focused on developing seeds, it is quite difficult to control and synchronize the development experimentally. As an alternative, we turned to suspension-cultured Catharanthus cells. When grown in Murashige and Skoog (MS) medium with 1.25 mM Pi, the cells depleted Pi in the medium and were effectively starved after 7 d (low-Pi cells) and contained negligible amounts of $InsP_6$ (Fig. 1, A and B). If the medium was supplemented with 7.5 mM Pi at day 3 and day 5, cells then accumulated high concentrations of both Pi and $InsP_6$ (high-Pi cells). While the concentration of $InsP_6$ continued to increase over the 7 d period (to 165.5 nmol g fresh weight $[FW]^{-1}$), the concentration of Pi peaked after 4 d (36.8 μmol g FW^{-1}) and thereafter remained constant. In addition to $InsP_6$, various other isomers of *myo*-inositol tetrakisphosphate ($InsP_4$) and *myo*-inositol pentakisphosphate ($InsP_5$) were detected in high-Pi cells (Fig. 1C), but *myo*-inositol monophosphate ($InsP_1$), *myo*-inositol bisphosphate ($InsP_2$), and *myo*-inositol trisphosphate ($InsP_3$) were either absent or below the detection limit. $InsP_6$ and other intermediates of Ins phosphate metabolism were measured using an ion chromatography method (see "Materials and Methods" and supplemental data).

We have also examined the accumulation of $InsP_6$ under high concentrations of applied Pi in suspension-cultured Arabidopsis cells. Unlike the case of Catharanthus, although $InsP_6$ accumulated in the high-Pi cells, the basal content of low-Pi cells was also higher, so the induction of $InsP_6$ synthesis in Arabidopsis cells was less obvious than in Catharanthus cells (Fig. 1D).

Cellular Localization of Ins Phosphates

The subcellular location of Ins phosphates in the cultured cells was investigated by comparing the profiles of Ins phosphates in protoplasts and in vacuoles isolated from the protoplasts. The measured contents were normalized using the activity of α-mannosidase, a vacuole-specific marker enzyme.

High-Pi cells were found to contain more than half of the $InsP_6$ in their vacuoles (Fig. 2A), while vacuoles from low-Pi cells accumulated very low levels of $InsP_6$ (Fig. 2B). Low levels of $InsP_4$s and $InsP_5$s were found in protoplasts of high-Pi cells. These peaks were confirmed to be Ins phosphates by the addition of phytase (phytase from *Aspergillus ficuum*, Sigma, St. Louis; Fig. 2C).

Effect of Cations on Accumulation of $InsP_6$ in Vacuoles

In mature dry seeds, $InsP_6$ is usually bound to K^+, Ca^{2+}, and Mg^2, forming phytin globoids. Thus, accumulation of $InsP_6$ might be closely related to storage of cations. The effects of cations on accumulation of $InsP_6$ were investigated by growing Catharanthus cells in high-Pi medium with Ca^{2+} (80 mM), Mg^{2+} (50 mM), Zn^{2+} (1 mM), or Mn^{2+} (3 mM) for 7 d. Concentrations of divalent cations were selected according to Hirschi et al. (2000). The amount of $InsP_6$ in cells incubated with Pi plus Ca^{2+} or Zn^{2+} increased markedly compared to that in cells supplied only with Pi, but decreased slightly in cells incubated with Mg^{2+} or Mn^{2+} (Fig. 3A). K^+ is a major monovalent cation detected in phytin globoids of some species (Otegui et al., 2002).

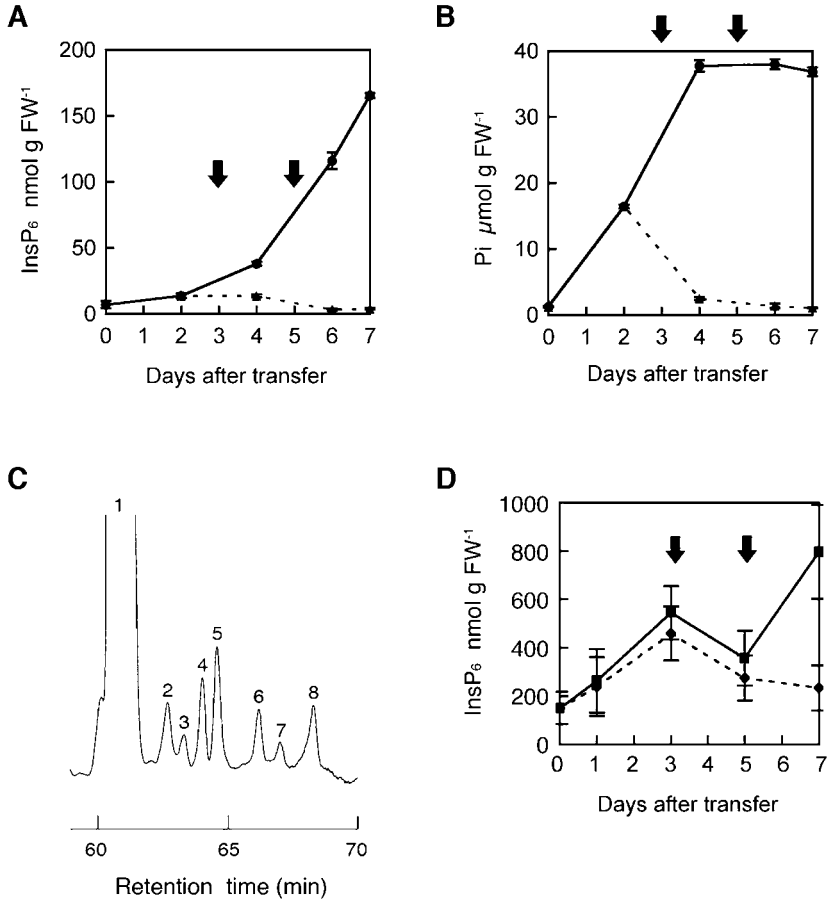

Figure 1. Changes in levels of $InsP_6$ and Pi in suspension-cultured Catharanthus cells grown with normal and high concentration of Pi. Low-Pi cell (represented by triangles and broken line) were grown with a normal MS salt containing 1.25 mM Pi for 7 d after transfer to fresh medium. High-Pi cells (represented by circles and straight line) were grown in MS salt with 2 times supplementation of 7.5 mM of Pi at days 3 and 5 (indicated by arrows). The 0, 2, 4, 6, and 7 d cells were harvested and homogenized in 2.4% (w/v) HCl. $InsP_6$ (A) and Pi (B) in the extracts were measured by ion chromatography. C, Assignment of Ins phosphates in high-Pi cells. Each peak was assigned as follows: 1, $InsP_6$; 2, Ins(2,4,5,6)P_4; 3, D/L-Ins(1,2,4,5,6)P_5; 4, Ins(1,2,3,4,6)P_5; 5, D/L-Ins(1,2,3,4,5)P_5; 6, D/L-Ins(1,4,5,6)P_4; 7, D/L-Ins(1,3,4,5)P_4; 8, D/L-Ins(1,2,4,5)P_4. D, Changes in levels of $InsP_6$ in suspension-cultured Arabidopsis cells grown with normal and high concentration of Pi. Low-Pi cell (represented by diamonds and broken line) were grown with a modified MS salt containing 3.75 mM Pi for 7 d after transfer to fresh medium. High-Pi cells (represented by squares and straight line) were grown in modified MS salt with 2 times supplementation of 7.5 mM of Pi at days 3 and 5 (indicated by arrows). The 0, 1, 3, 5, and 7 d cells were harvested and homogenized in 2.4% (w/v) HCl.

Addition of 125 mM K^+ in high-Pi increased $InsP_6$ markedly, but 75 mM K^+ cells showed levels comparable to that in high-Pi cells (Fig. 3A). The Pi concentration in each treatment was almost unchanged, except for high-Pi + Ca^{2+} cells in which the Pi concentration increased 2-fold (Fig. 3B).

The location of $InsP_6$ in Catharanthus cells was investigated by isolating protoplasts and vacuoles from cells treated in high Pi together with Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, or K^+. In high-Pi + Ca^{2+}, + Mg^{2+}, + Mn^{2+}, or + K^+[125] cells, $InsP_6$ was mainly accumulated in vacuoles (Fig. 4). By contrast, in high-Pi + Zn^{2+} cells, $InsP_6$ was predominantly accumulated in non-vacuolar compartments. In high-Pi + Zn^{2+} cells, Zn^{2+} level in whole protoplasts was 17.5 ± 3.1 nmol α-mannosidase activity^{-1}, while in vacuoles was 4.7 ± 1.0 nmol α-mannosidase activity^{-1}. Zn^{2+} level in whole protoplasts was 3.7-fold more than vacuoles. This indicates that Zn^{2+} was mostly in the extravacuolar space. In order to analyze the further localization of $InsP_6$, we fractionated cell homogenates with centrifugation (Table I). When homogenates of high-Pi + Ca^{2+} cells were centrifuged at 3,000*g*, $InsP_6$ was recovered in a soluble fraction (S 3,000*g*) including membranous organelles. But when homogenates of high-Pi + Zn^{2+} cells were centrifuged, $InsP_6$ was mainly detected in the buffer-insoluble pellet (P 3,000*g*), and 28.8% of total Zn^{2+} was also in the pellet, suggesting that $InsP_6$ bound to Zn^{2+} formed insoluble aggregates within the cytosol. These results suggest that $InsP_6$ is synthesized in cytosol and then incorporated into vacuoles.

The mechanism for transport of $InsP_6$ into the vacuole has so far remained unresolved, so the effects of brefeldin A (an inhibitor of vesicle transport), wortmannin (an inhibitor of phosphoinositide metabolism), or monensin (an inhibitor of membrane transport) on $InsP_6$ synthesis in high-Pi cells were examined. When wortmannin (5 μM) was added after 6 d to high-Pi cells, the level of $InsP_6$ after 7 d cells had decreased to 40% of that in control high-Pi cells (Table II). Brefeldin A (5 μM) or monensin (2.5 μM) caused 3.5- and 1.8-fold increases in $InsP_6$, respectively, compared to that in high-Pi cells. Changes in levels of Pi following addition of these inhibitors were much smaller than that of $InsP_6$. The brefeldin A-treated high-Pi cells accumulated 38.0 ± 10.6 nmol α-mannosidase activity^{-1} of $InsP_6$ in whole protoplast and 31.1 ± 10.7 nmol α-mannosidase activity^{-1} of $InsP_6$ (81.8% of total $InsP_6$) in their vacuole.

Analysis of $InsP_6$ Synthesis in Catharanthus Cells

The level of MIPS in Catharanthus was investigated with a specific antibody against a 62-kD recombinant

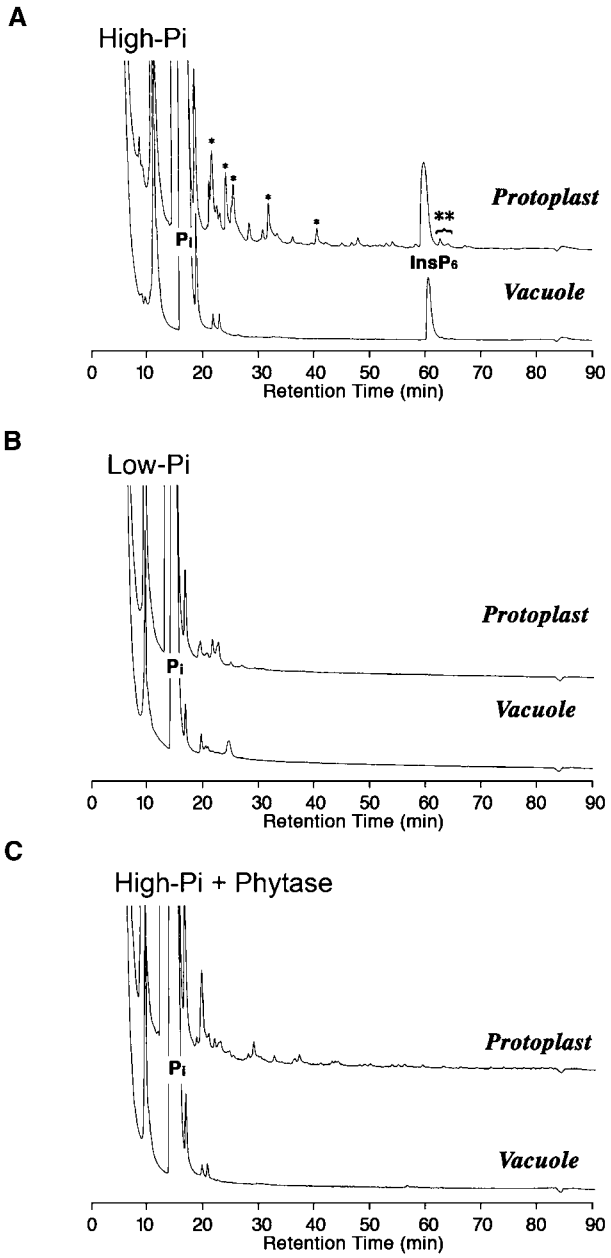

Figure 2. A, Distribution of Ins phosphates in protoplasts and vacuoles from high-Pi cells. Double asterisks indicate mixtures of $InsP_4$s and/or $InsP_5$s shown in Figure 1C. B, Absence of Ins phosphates in protoplasts and vacuoles from low-Pi cells. C, Changes in levels of Ins phosphates following addition of phytase to extracts. The asterisks in A indicate unassigned peaks that were diminished by phytase. The extracts were normalized to 20 mU α-mannosidase activity.

Arabidopsis MIPS (At4g39800) protein. The antibody showed cross reactivity to a 56-kD Catharanthus protein (Fig. 5A), whose expression was reduced by addition of Ins in a dose-dependent manner (Fig. 5A, lanes 8 and 9). The Catharanthus MIPS homolog was found to be constitutively expressed in 7-d-old cells in all of the treatments examined here (Fig. 5A, lanes 2–7). Likewise, K^+ did not alter the level of MIPS (data not shown). Thus, increase of $InsP_6$ content and/or the promotive effect by metal cations were not caused by induction of MIPS.

Ins(1,4,5)P_3 6-/3-kinase (Ipk2) produces Ins(1,3,4,5,6)P_5 from D-Ins(1,4,5)P_3 by its dual-kinase activity. This enzyme is more specific to $InsP_6$ synthesis than MIPS. Immunoblot analysis for D-Ins(1,4,5)P_3 kinase (Ipk2), using a specific antibody against a recombinant protein, Arabidopsis AtIpk2α (At5g07370), fused with thioredoxin, revealed that the antibody recognized a 53-kD thioredoxin-AtIpk2α fusion protein (Fig. 5B, lane 10) and a 33-kD Catharanthus protein (Fig. 5B, lanes 2–9). This kinase existed both in low-Pi and high-Pi conditions at the same level and decreased following addition of Ins, as also occurred with MIPS. Thus, the level of $InsP_6$ does not appear to relate to the levels of either Ins(1,4,5)P_3 6-/3-kinase or MIPS. We also conducted an immunoblot analysis of Ins(1,3,4)P_3 5-/6-kinase using an antibody against Arabidopsis Ins(1,3,4)P_3 5-/6-kinase (At4g39800) protein fused with thioredoxin. A positive signal could not be detected, indicating low cross reactivity of the antibody to and/or low induction level of this kinase in the Catharanthus cells. Brefeldin A, wortmannin, or monensin did not change the levels of MIPS and Ipk2 proteins (data not shown).

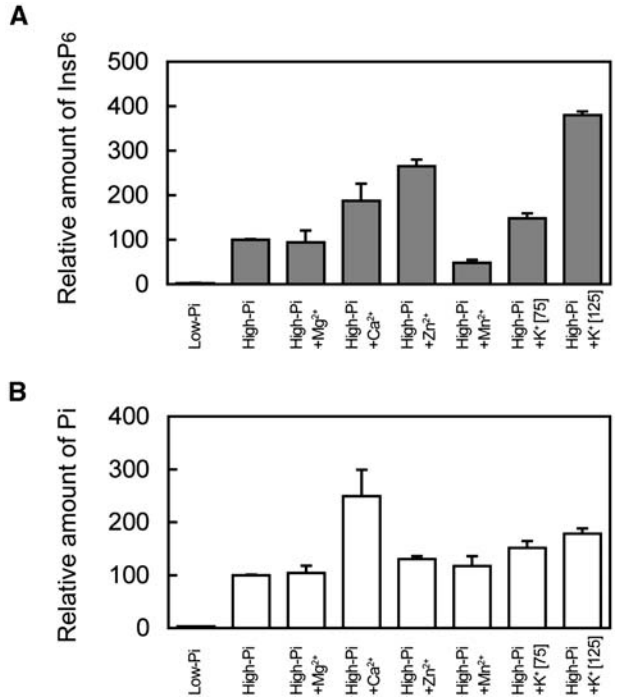

Figure 3. Effect of metal cations on $InsP_6$ synthesis induced by high concentration of Pi. Catharanthus cells were grown under high-Pi with 50 mM $MgCl_2$, 80 mM $CaCl_2$, 1 mM $ZnCl_2$, 3 mM $MnCl_2$, 75 mM KCl, or 125 mM KCl. The 7-d cells were harvested and homogenized in 2.4% (w/v) HCl. $InsP_6$ (A) and Pi (B) in the extracts were measured by ion chromatography. Each value is represented as percentage of relative content to high-Pi cells. Data are average of at least three experiments.

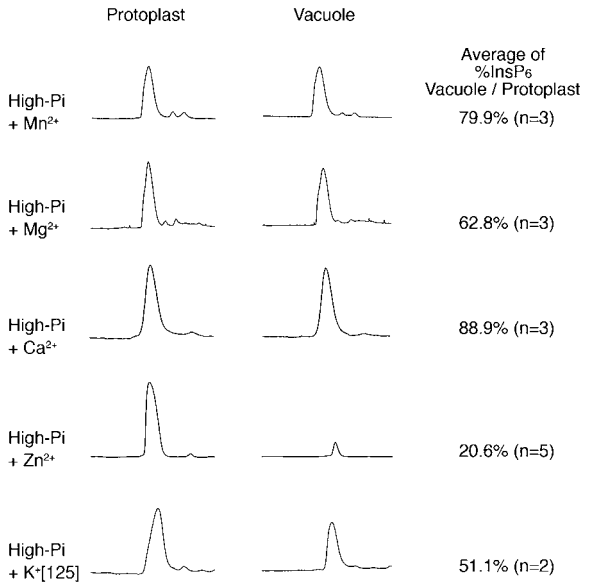

Figure 4. Effect of metal cations on accumulation of $InsP_6$ in vacuoles. Protoplasts and vacuoles were isolated from 7-d Catharanthus cells grown under high-Pi with 50 mM $MgCl_2$, 80 mM $CaCl_2$, 1 mM $ZnCl_2$, 3 mM $MnCl_2$, or 125 mM KCl. The amounts of $InsP_6$ in protoplasts and vacuoles were normalized to 20 mU of α-mannosidase activity to enable comparisons. Data show typical chromatograms in more than three independent experiments. The means of $InsP_6$ amounts occupied in vacuoles versus protoplasts were shown in the right.

DISCUSSION

$InsP_6$ Synthesis and Accumulation in the Vacuole

The use of suspension-cultured Catharanthus cells provides an experimental system for investigating $InsP_6$ synthesis in vivo. Accumulation of $InsP_6$ in the vacuole was easily inducible in these cells when grown with high concentration of Pi (Fig. 2A). Catharanthus cells can grow in the medium (1.25 mM Pi) without Ins (see "Materials and Methods"). By contrast, Arabidopsis cells need the medium containing Ins and higher Pi (3.75 mM). These differences may influence levels of $InsP_6$ synthesis in both cells (Fig. 1, A and D). Thus, we concluded that Catharanthus cells are more suitable material for investigating $InsP_6$ synthesis than Arabidopsis cells.

Various isomers of $InsP_4$ and $InsP_5$ were also detected in Catharanthus cells (Fig. 1C). Part of them was also detected in vacuoles (Figs. 2A and 4). However, these may be degraded products of $InsP_6$. $InsP_1$ to $InsP_3$ were not detected, which may indicate their rapid sequential phosphorylation into higher Ins phosphates without the accumulation of intermediates.

The actual site of $InsP_6$ synthesis is unknown, although the requirement for ATP for the phosphorylation steps from $InsP_1$ to $InsP_6$ strongly points to a cytosolic location. Indeed, MIPS localizes to the whole cytoplasm in *Phaseolus vulgaris* (Lackey et al., 2003). The questions that need solutions are (1) what regulates the synthesis of $InsP_6$, and (2) how is it transported to the vacuole? In relation to the first question, there is clearly some association between cellular Pi and $InsP_6$ (Fig. 1) and, as we have shown, between $InsP_6$ and certain metals (Fig. 3). Ca^{2+}, Zn^{2+}, and Mg^{2+} are known to form insoluble precipitates with $InsP_6$ (Urbano et al., 2000), and therefore compartmentation of these metals following uptake will be important. At a high concentration (125 mM), K^+ induced an increase in $InsP_6$ (Fig. 3), but this might be the result of osmotic stress, and the synthesis of $InsP_6$ may be to provide a compatible osmotic solute to counter this stress. The concentration of Ca^{2+} in the cytosol is maintained at submicromolar levels, although Ca^{2+} can reach much higher levels in the vacuole (our data suggest 2–3 mM in Catharanthus; data not shown). It is therefore not surprising that under high Ca^{2+} conditions, almost all $InsP_6$ was detected in the vacuole (Fig. 4). The finding that under high Zn^{2+} supply most $InsP_6$ occurred in the cytosol and that a significant proportion could be recovered in an insoluble fraction (Fig. 4; Table I) seems to indicate that the concentration of Zn^{2+} in the cytosol under these conditions is high enough to induce precipitation. An in vitro analysis showed that 200 μM $InsP_6$ was completely precipitated by addition of 1 mM $ZnCl_2$ or $CaCl_2$ at pH 7.2 (data not shown). This could possibly be due to the reduction in $InsP_6$ free acid caused by increased precipitation stimulating de novo synthesis of $InsP_6$ via sequential enzymatic equilibrium. Recently, cellular functions for $InsP_6$ in plants other than storage of Pi have been proposed, such as an abscisic acid-induced Ca^{2+} release in guard cells (Lemtiti-Chieh et al., 2003) or a relationship to turion formation in duckweed (*Spirodela polyrrhiza*; Flores and Smart, 2000). Under these circumstances, it may be necessary to compensate for the loss of $InsP_6$ due to chelation in order to maintain an appropriate level of free $InsP_6$ to regulate these other activities.

Table I. *Centrifugation analysis of soluble and insoluble forms of $InsP_6$*

High-Pi + Ca^{2+} and high-Pi + Zn^{2+} cells were homogenized and centrifuged at 3,000*g* and the $InsP_6$ and zinc contents of each supernatant (S 3,000*g*) and precipitate (P 3,000*g*) were measured. Results are means ± SE (n = 3). –, Not detected.

	$InsP_6$		Pi		Zn^{2+}	
	S 3,000*g*	P 3,000*g*	S 3,000*g*	P 3,000*g*	S 3,000*g*	P 3,000*g*
	nmol g FW^{-1}		*μmol g FW^{-1}*		*μmol g FW^{-1}*	
High-Pi + Ca^{2+}	286.3 ± 57.8	49.8 ± 7.6	74.2 ± 12.2	8.9 ± 1.8	–	–
High-Pi + Zn^{2+}	67.5 ± 27.9	355.5 ± 113.8	41.5 ± 9.9	8.4 ± 2.1	0.42 ± 0.09	0.17 ± 0.02

Table II. *Effect of various inhibitors on $InsP_6$ synthesis*

High-Pi cells were cultured for 6 d, then each inhibitor was added into the culture and incubated for 24 h. Results are means ± SE ($n = 3$).

	$InsP_6$ (% of High-Pi)	Pi (% of High-Pi)
High-Pi + dimethyl sulfoxide	99.8 ± 4.2	106.0 ± 5.5
High-Pi + brefeldin A	353.3 ± 42.7	103.8 ± 1.9
High-Pi + wortmannin	43.8 ± 2.9	107.3 ± 3.1
High-Pi + monensin	176.8 ± 17.1	128.0 ± 3.6

Metabolic Regulation of Ins Phosphates Synthesis

In Arabidopsis, three genes of MIPS (At2g22240, At4g39800, and At5g10170) are highly conserved and the amino acid identities are >89%. Since the present antibody is polyclonal, MIPS expressed by genes other than At4g39800 might be detected. Although we do not know how many MIPS genes are in Catharanthus genome, it is likely that the antibody detected most MIPS expressed in suspension-cultured Catharanthus cells. AtIpk2β is localized in nuclei and catalyzes the conversion from D-Ins(1,4,5)P_3 to D-Ins(1,3,4,5,6)P_5 (Xia et al., 2003). We have shown that the level of Ins(1,4,5)P_3 kinase (Ipk2), as well as MIPS (Fig. 5), did not noticeably differ under conditions of either high or low $InsP_6$ accumulation, and is therefore unlikely to be a candidate for the regulation of synthesis of $InsP_6$ in the present condition. Addition of Ins suppressed both enzymes (Fig. 5). Suppression of MIPS gene in the presence of Ins has been well known (Johnson and Sussex, 1995), but Ins-dependent suppression of Ipk2α may be the first report. Although in this condition (with Ins and without Pi) $InsP_6$ was not accumulated, the regulation mechanism of those enzymes by Ins and Pi is a future subject. As yet it is not clear whether expression level of Ins(1,3,4) 5-/6-kinase is very weak or the antibody used here has no cross reactivity to the kinase protein in Catharanthus cells. We should improve the analytical conditions to investigate this kinase in detail.

The mechanism by which $InsP_6$ is transported to the vacuole remains to be resolved. It has been suggested that $InsP_6$ may be transported from ER lumen to protein storage vacuoles via a vesicle transport pathway (Greenwood and Bewley, 1984). This hypothesis was partly supported by the brefeldin A experiments (Table II), but the incorporation of $InsP_6$ into the ER lumen was not demonstrated. Brefeldin A had a strong stimulatory effect on $InsP_6$ synthesis and its accumulation in the vacuole. This would seem hard to reconcile with the expected inhibition of vesicle transport by brefeldin A, but it is known that brefeldin A has a range of effects on the endomembrane system, some of which (e.g. tubulation of Golgi) may in fact facilitate transfer to the vacuole (Klausner et al., 1992). Wortmannin and monensin also affected $InsP_6$ synthesis, suggesting phosphoinositide metabolism and membrane transport are also related to $InsP_6$ synthesis.

In conclusion, the suspension-culture system described here has many advantages for investigating the regulation of synthesis and compartmentation of $InsP_6$. Additionally, the improvements to the detection system allow quantitative measurements of the key intermediates of $InsP_6$ metabolism. With the increasing efforts to produce genetically modified plants containing lower levels of Ins phosphates or for environmental phytoremediation (Raboy, 2001; Brinch-Pedersen et al., 2002), these research technologies will be useful.

MATERIALS AND METHODS

Ins Phosphates Measurement

For Ins phosphates and Pi measurements, a DX-500 ion chromatography system (Dionex, Osaka) consisting of a gradient pump, a 25-μL sample loop, and a conductivity detector was used as described previously (Baluyot and Hartford, 1996; Sekiguchi et al., 2000). Dionex IonPac AS11 (2 mm i.d. × 250 mm)

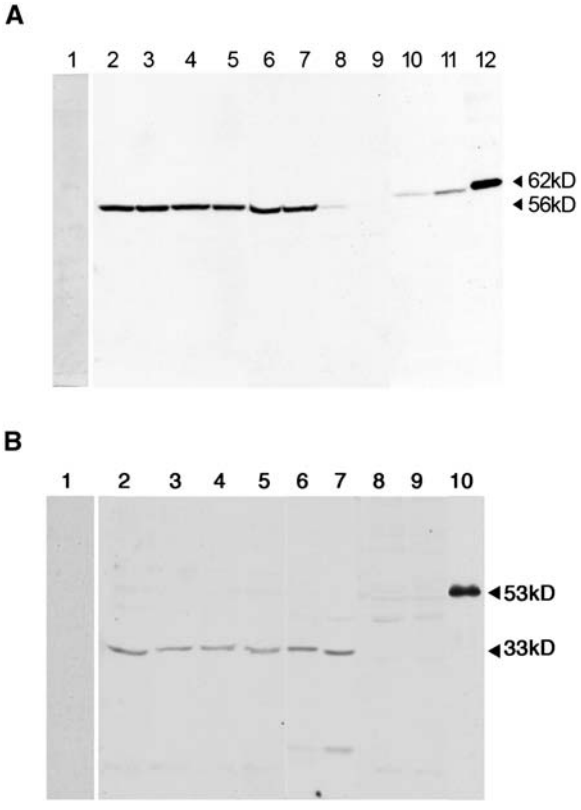

Figure 5. Immunoblot analyses for (A) MIPS and (B) Ins(1,4,5)P_3 kinase (Ipk2) in Catharanthus cells grown under various conditions. Immunoblots with preimmune (A and B, lane 1), anti-MIPS (A, lanes 2–12), or anti-Ipk2 (B, lanes 2–9) are shown. The lanes show total protein (80 μg) from protoplasts of low-Pi (A and B, lane 2), high-Pi (A and B, lane 3), high-Pi + 80 mM $CaCl_2$ (A and B, lane 4), high-Pi + 1 mM $ZnCl_2$ (A and B, lane 5), high-Pi + 50 mM $MgCl_2$ (A and B, lane 6), or high-Pi + 3 mM $MnCl_2$ (A and B, lane 7); total proteins (80 μg) from cells of +10 mM Ins (A and B, lane 8), +50 mM Ins (A and B, lane 9); total proteins (10 μg) from mature leaves (A, lane 10) and immature seeds (A, lane 11) of Arabidopsis; recombinant MIPS protein (0.08 μg; A, lane 12); or recombinant thioredoxin-AtIpk2α fusion protein (0.2 μg; B, lane 10).

and IonPac AG11 (2 mm i.d. × 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 i.d. × 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. The current method could separate $InsP_1$ to $InsP_6$ in a single gradient elution. Twenty-five microliters of the filtered samples were automatically injected by an auto-sampler 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 $InsP_6$ would be less than 50 nM. Our system could not distinguish enantiomers, but many isomers could be separated among 64 species of Ins phosphates; we could finally separate 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 6 $InsP_5$ isomers independently (see supplemental material).

Reagents

$InsP_6$ was purchased from Sigma. All synthetic isomers of $InsP_1$s (Chung and Chang, 1996a), $InsP_2$s (Chung et al., 1998), $InsP_3$s (Chung et al., 1996), $InsP_4$s (Chung and Chang, 1995), and $InsP_5$s (Chung and Chang, 1996b) were synthesized, and their structures and purities were confirmed in Dr. Sung-Kee Chung's laboratory (Pohang University of Science and Technology, Korea).

Plant Materials

Catharanthus roseus L. G. Don cells were cultured in 20 mL of MS medium, pH 6.2, supplemented with 4 μM nicotinic acid, 2.5 μM pyridoxine, 0.3 μM thiamine, 20 μM Gly, 4.5 μM 2,4-dichlorophenoxyacetic acid, and 3% (w/v) Suc (MS medium). Cells were cultured with shaking at 26°C under dim light and transferred to fresh medium every 7 d.

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 suspension cells were cultured in 20 mL of MS medium, pH 6.2, supplemented with 2.5 mM KH_2PO_4, 1 mM Ins, 16 μM nicotinic acid, 10 μM pyridoxine, 60 μM thiamine, 4.5 μM 2,4-dichlorophenoxyacetic acid, and 3% (w/v) Suc (modified MS medium). Cells were cultured with shaking at 23°C under dim light and transferred to fresh medium every 7 d.

For treatment with high concentrations of Pi, 7.5 mM KH_2PO_4 was added twice (3 and 5 d) during the 7 d culture. For treatment with high-metal cations, 25 mM $MgCl_2$, 40 mM $CaCl_2$, 0.5 mM $ZnCl_2$, 1.5 mM $MnCl_2$, or 27.5 mM or 52.5 mM KCl were added twice (3 and 5 d) with 7.5 mM Pi in the culture medium. To investigate the effect of various inhibitors on $InsP_6$ synthesis, 6-d cells cultured in high concentration of Pi were treated for 24 h with 5 mM of Brefeldin A (Wako Pure Chemical Industries, Osaka), 5 mM of wortmannin (Wako), or 2.5 mM of monensin (Sigma). Each chemical was dissolved in dimethyl sulfoxide as stock solutions (×1,000).

To determine Pi and Ins phosphates contents, harvested cells were ground with a mortar and a pestle in liquid nitrogen and homogenized in 2.4% (w/v) HCl. The homogenates were boiled for 10 min and centrifuged at 20,000*g* for 10 min at 4°C. The supernatant was filtered through a 0.45-μm filter (Ekicro-disc AcroLC, Pall Gelman Laboratory, Tokyo) and was diluted with deionized water, then 25 μL of the filtrate was subjected to ion chromatography. The HCl-extracts were kept for up to 48 h at 37°C, then the samples were measured. The peak area of $InsP_6$ was not affected by storage for up to 48 h (data not shown).

Isolation of Protoplasts and Vacuoles

Protoplasts and vacuoles were isolated as described previously (Massonneau et al., 2000; Shimaoka et al., 2004). For analysis of contents, protoplasts and vacuoles were freeze-thawed. A part of them was treated with 2.4% (w/v) HCl for measurement of Ins phosphates, as described above.

Assay of α-Mannosidase Activity

Freeze-thawed protoplasts and vacuoles were subjected to assay of α-mannosidase activity. Two milligrams of *p*-nitrophenyl-α-mannopyranoside were dissolved in 1 mL of dimethylformamide (100 × substrate). Each 100 μL of sample was mixed with 400 μL of 1 × substrate diluted by 100 mM sodium-citrate buffer, pH 5.6, otherwise with citrate buffer only. After incubation for 1 h at 37°C, the reaction was stopped by addition of 1 mL of 200 mM Na_2CO_3. Samples were centrifuged at 5,800*g* for 2 min, and the absorbance of each supernatant was measured at 405 nm. One unit of α-mannosidase activity was defined by one absorbance unit at 405 nm.

Measurement of $InsP_6$ and Pi in Soluble and Insoluble Fraction of Catharanthus Cells

Seven-day-old Catharanthus cells were sonicated (S-250D; Branson, Danbury, CT) in four volumes of buffer A composed of 10 mM Tris-HCl, pH 7.5, with 13% (w/v) Suc. The homogenate was centrifuged at 3,000*g* for 10 min at 4°C. The centrifuged supernatant and precipitate resuspended in buffer A were collected and subjected to measurement of $InsP_6$ and Pi as described above.

Measurement of Zinc

Intracellular zinc content was measured with Zincon (Dojindo, Tokyo). Forty microliters of 1 mM Zincon was added into 200 μL of samples diluted in 50 mM Tris-HCl, pH 8.0, and absorbance of the samples was measured immediately at 620 nm.

Preparation for Antibodies against MIPS and Ins(1,4,5)P_3 Kinase

Arabidopsis expressed sequence tag clones (accession nos. AV525103 and AV528014) for an Arabidopsis MIPS gene (At4g39800) and an Arabidopsis Ins(1,4,5)P_3 kinase gene (At5g07370), respectively, were provided from Kazusa DNA Research Institute, Chiba, Japan (http://www.kazusa.or.jp/). For a MIPS gene, two primers, 5′-GAATTCATGTTTATTGAGAGCTTCAAAGTT-3′ and 5′-CTCGAGCTTGAACTCCATGATCATGTTGTT-3′, were designed on the basis of N-terminal and C-terminal sequences of At4g39800, respectively. The amplified DNA was digested by *Xho*I and *Eco*RI and inserted into a *Xho*I-*Eco*RI site of pET21a vector (EMD Biosciences, San Diego). The ligated At4g39800-pET21a plasmid was introduced into *Escherichia coli* BL21(DE3) strain (EMD Biosciences). For an Ins(1,4,5)P_3 kinase gene, two primers, 5′-GGATCCATGCAGCTCAAAGTCCCTGAACAT-3′ and 5′-GTCGACCTAA-GAATCTGCAGACTCATCTGG-3′, were designed on the basis of N-terminal and C-terminal sequences of At5g07370, respectively. The amplified DNA was digested by *Bam*HI and *Sal*I and inserted into the *Bam*HI-*Sal*I site of pET32a vector (EMD Biosciences). The ligated At5g07370-pET32a plasmid was introduced into *E. coli* BL21(DE3) strain (EMD Biosciences). The recombinant proteins were purified via a 6 × His-tag by using HiTrap Chelating HP column (Amersham Biosciences, Piscataway, NJ) and used as antigens. Specific antisera were provided by Shibayagi (Gunma, Japan).

Immunoblot Analysis

Crude extracts were prepared from Arabidopsis plants and Catharanthus protoplasts according to the following procedures. Immature seeds in green siliques (grown for 4 weeks) and mature leaves (grown for 4 weeks) of Arabidopsis or protoplasts isolated from 7-d-old Catharanthus cells were ground with a mortar and pestle in liquid nitrogen and resuspended with 10 mM Tris-HCl, pH 7.5. All samples were subjected to SDS-PAGE with 7.5% or 10% (w/v) acrylamide gel and electrically transferred to a polyvinylidene difluoride membrane (Bio Craft, Tokyo). The membrane blot was incubated with specific antibodies against Arabidopsis MIPS or Ins(1,4,5)P_3 kinase. Horseradish peroxidase-conjugate antibodies raised in donkey against rabbit IgG (Amersham Biosciences) were used as secondary antibodies. Immuno-detection was performed with an enhanced chemiluminescence kit (an ECL system, Amersham Biosciences) according to the manufacturer's directions.

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