Up-Regulation of Phosphoinositide Metabolism in Tobacco Cells Constitutively Expressing the Human Type I Inositol Polyphosphate 5-Phosphatase[1]

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To evaluate the impact of suppressing inositol 1,4,5-trisphosphate ($InsP_3$) in plants, tobacco (*Nicotiana tabacum*) cells were transformed with the human type I inositol polyphosphate 5-phosphatase (InsP 5-ptase), an enzyme which specifically hydrolyzes $InsP_3$. The transgenic cell lines showed a 12- to 25-fold increase in InsP 5-ptase activity in vitro and a 60% to 80% reduction in basal $InsP_3$ compared with wild-type cells. Stimulation with Mas-7, a synthetic analog of the wasp venom peptide mastoparan, resulted in an approximately 2-fold increase in $InsP_3$ in both wild-type and transgenic cells. However, even with stimulation, $InsP_3$ levels in the transgenic cells did not reach wild-type basal values, suggesting that $InsP_3$ signaling is compromised. Analysis of whole-cell lipids indicated that phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$), the lipid precursor of $InsP_3$, was greatly reduced in the transgenic cells. In vitro assays of enzymes involved in $PtdInsP_2$ metabolism showed that the activity of the $PtdInsP_2$-hydrolyzing enzyme phospholipase C was not significantly altered in the transgenic cells. In contrast, the activity of the plasma membrane PtdInsP 5 kinase was increased by approximately 3-fold in the transgenic cells. In vivo labeling studies revealed a greater incorporation of ^{32}P into $PtdInsP_2$ in the transgenic cells compared with the wild type, indicating that the rate of $PtdInsP_2$ synthesis was increased. These studies show that the constitutive expression of the human type I InsP 5-ptase in tobacco cells leads to an up-regulation of the phosphoinositide pathway and highlight the importance of $PtdInsP_2$ synthesis as a regulatory step in this system.

In plants the phosphoinositide (PI) pathway has been implicated in the transduction of signals after a multitude of stimuli (Drøbak, 1992; Munnik et al., 1998a; Stevenson et al., 2000). Upon stimulation, the membrane-associated phospholipid, phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$), is hydrolyzed by phospholipase C (PLC) to produce inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (Berridge, 1993). $InsP_3$ can act as a soluble second messenger to mediate the release of Ca^{2+} from intracellular stores, such as the vacuole (for review, see Sanders et al., 1999). Furthermore, because $InsP_3$ can travel between cells through plasmodesmatal connections (Tucker and Boss, 1996), it fulfills the requirements for a rapidly diffusible signaling molecule in both intra- and intercellular signal propagation.

Rapid transient increases in $InsP_3$ have been demonstrated in various plant tissues in response to environmental stimuli and chemical effectors, including hyperosmotic stress (Srivastava et al., 1989; Heilmann et al., 1999, 2001; DeWald et al., 2001; Takahashi et al., 2001), salinity (Drøbak and Watkins, 2000), cold shock (Smolenska-Sym and Kacperska, 1996), oligogalacturonide elicitors (Legendre et al., 1993), fusicoccin (Aducci and Marra, 1990), mastoparan (Legendre et al., 1993; Drøbak and Watkins, 1994; Cho et al., 1995; Franklin-Tong et al., 1996), abscisic acid (ABA; Lee et al., 1996), and gravity (Perera et al., 1999, 2001).

In addition to a second messenger role for $InsP_3$ in reversible turgor-driven processes, such as regulation of stomatal aperture and cellular osmotic homeostasis, evidence is accumulating that long-term $InsP_3$ changes may be involved in directing differential plant growth (for review, see Stevenson et al., 2000). Studies of tip-growing cells such as pollen tubes (Franklin-Tong et al., 1996; Kost et al., 1999), and the graviresponsive pulvinal cells of cereal grasses (Perera et al., 1999, 2001) have shown that long-term increases in both $InsP_3$ and $PtdInsP_2$ synthesis are involved in the regulation of cell elongation.

For $InsP_3$ to function as a second messenger, the duration of an $InsP_3$ signal must be tightly regulated and the signal rapidly degraded to affect spatially and temporally discrete responses. In animal cells, removal of $InsP_3$ can occur either via the phosphorylation of the D-3 position of the inositol ring by a specific $Ins(1,4,5)P_3$ 3-kinase to form $InsP_4$ (Takazawa et al., 1991) or by dephosphorylation of the D-5 po-

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sition of the inositol ring by inositol polyphosphate 5-phosphatase (InsP 5-ptase) to form $InsP_2$.

The mammalian InsP 5-ptases are a family of enzymes classified into four types based on their substrate specificity (Mitchell et al., 1996; Jefferson et al., 1997; Majerus et al., 1999). The type I InsP 5-ptase exclusively hydrolyzes the water-soluble inositol polyphosphates, $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$, unlike the type II and III InsP 5-ptases, which can hydrolyze both the water soluble and the lipid inositol polyphosphates (Laxminarayan et al., 1993, 1994; Matzaris et al., 1994) and the type IV enzymes that only hydrolyze the lipid $PtdIns(3,4,5)P_3$ (Majerus et al., 1999). The mammalian type I InsP 5-ptase, which is encoded by a single gene (Mitchell et al., 1996; Erneux et al., 1998), plays a major role in the termination of $InsP_3$ signals in animal cells and over- and underproduction of this enzyme was shown to profoundly affect $InsP_3$-mediated Ca^{2+} signaling (De Smedt et al., 1997; Speed et al., 1996, 1999; Speed and Mitchell, 2000).

At present, the mechanisms of $InsP_3$ hydrolysis in plants are not well understood. There has been no biochemical evidence of $InsP_3$ 3-kinases in plants (Phillippy, 1999; Brearley and Hanke, 2000). However, there are many reports of InsP phosphatases, suggesting that $InsP_3$ degradation in plants is primarily via dephosphorylation. Early biochemical studies (Joseph et al., 1989; Drøbak et al., 1991; Martinoia et al., 1993) have implicated the involvement of both an InsP 5-ptase and InsP 1-ptase in $InsP_3$ hydrolysis. The principal route of $InsP_3$ inactivation may involve either an InsP 1-ptase or an InsP 5-ptase depending on the specific plant system or developmental stage (Brearley et al., 1997; DePass et al., 2001). At the molecular level, the Arabidopsis genome contains 15 putative InsP 5-ptase genes, which can be categorized into three groups based on size and sequence (Berdy et al., 2001). Our current understanding of the function and localization of the plant InsP 5-ptases is limited. Two of the Arabidopsis InsP 5-ptase genes, At5PTase1 (Berdy et al., 2001) and AtIP5PII (Sanchez and Chua, 2001), have been characterized and shown to possess $InsP_3$-hydrolyzing activity in vitro and in vivo. Both At5PT1 and AtIP5PII share significantly greater sequence similarity over their catalytic domains with the animal type II InsP 5-ptase enzymes (42% and 57%, respectively) than with the animal type I enzyme (approximately 19%). Based on sequence analysis, there does not appear to be a homolog of the human type I InsP 5-ptase in the Arabidopsis genome.

Although changes in $InsP_3$ may be an essential component of the PI signaling pathway, it has been difficult to link these changes with a specific physiological response. Pharmacological agents such as the aminosteroid PLC inhibitor U73122 have been used quite effectively to interfere with $InsP_3$ production and thereby block a downstream response in certain plant systems (Staxen et al., 1999; Perera et al., 2001; Takahashi et al., 2001). However, this approach has its limitations, primarily due to problems with uptake and delivery of compounds into intact plant tissues, and the potential nonspecific side effects of the inhibitor treatments (Cho et al., 1995; Munnik et al., 1998a). A molecular approach to reduce or eliminate $InsP_3$ would have broader applicability and the InsP 5-ptase enzyme is an obvious target for manipulation.

Recently, Sanchez and Chua (2001) have examined the downstream consequences of altering $InsP_3$ in ABA-regulated processes by generating transgenic plants expressing an Arabidopsis InsP 5-ptase gene (AtIP5PII) under the control of an inducible promoter. A 4- to 5-fold increase in InsP 5-ptase activity was detected in the transgenic plants after approximately 12 h of induction, and transgenic lines exhibited decreased sensitivity to the inhibitory effects of ABA on seed germination and growth, seen in wild-type plants. Expression of ABA-responsive genes after 4 h of ABA treatment was also attenuated in the transgenic plants.

The goal of our work was to study the effects of constitutively lowering cellular $InsP_3$ on PI signaling and metabolism in plant cells. The human type I InsP 5-ptase was selected for this study because it has an approximately two orders of magnitude higher specific activity for $Ins(1,4,5)P_3$ hydrolysis than the plant InsP 5-ptase enzymes characterized to date.

Here, we report that the human type I InsP 5-ptase is expressed in tobacco (*Nicotiana tabacum*) suspension cells and that the protein is active. The expression of InsP 5-ptase in tobacco cells leads to a drastic reduction in the basal levels of $InsP_3$ and in an attenuation of stimulus-induced $InsP_3$ changes. Significantly, the constitutive expression of InsP 5-ptase affected the upstream components of the PI pathway, resulting in increased PtdInsP 5 kinase activity and $PtdInsP_2$ biosynthesis.

RESULTS

Expression of the Type I InsP 5-ptase Gene in Transgenic Tobacco Cells

Tobacco (NT1) cells in suspension culture were transformed with the 35S promoter::InsP 5-ptase construct shown in Figure 1A. Of 15 independent transgenic microcalli tested for the presence of the InsP 5-ptase protein, significant protein expression was detected in 11 lines (data not shown). Three tobacco cell lines transformed with the InsP 5-ptase (I2-2, I2-8, and I2-11) and two control lines transformed with the 35S promoter-containing binary vector alone (C-3 and C-5) were chosen for further analysis. Figure 1B shows a blot of total RNA prepared from cells harvested on d 4 of the culture cycle, probed with the InsP 5-ptase cDNA. An approximately 1.7-kb transcript indicative of transgene expression was detected only in the cell lines transformed with InsP 5-ptase. No transcript was detected in wild-type and

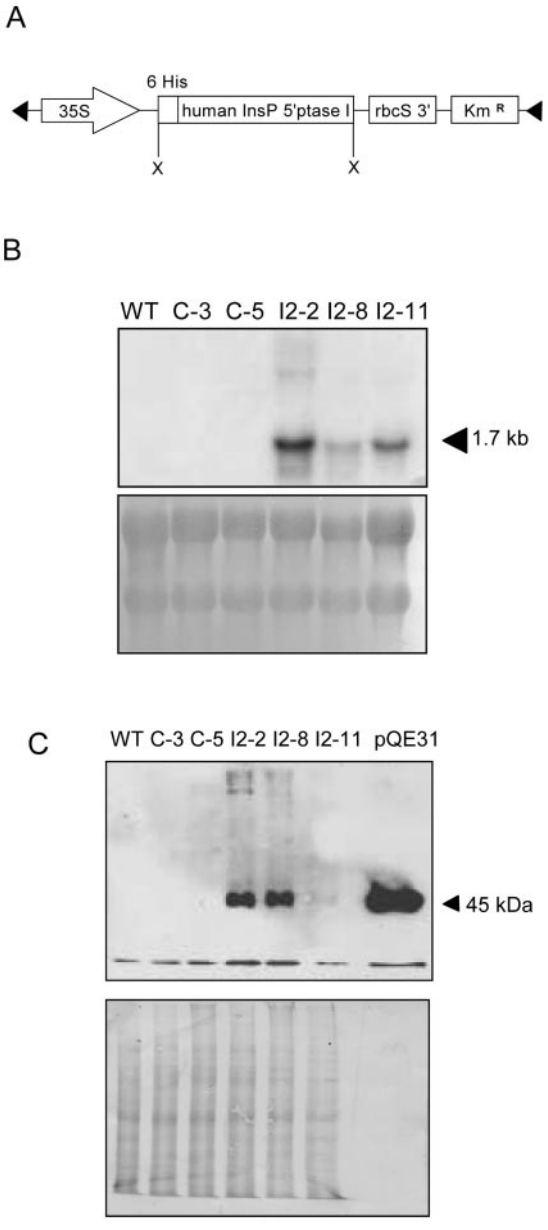

Figure 1. Expression of the human InsP 5-ptase gene in the transgenic tobacco cells. A, Schematic of the binary plasmid containing the InsP 5-ptase construct used to transform tobacco cells. 35S, Cauliflower mosaic virus 35S promoter; 6 His, His tag; human InsP 5-ptase I, coding region of the human type I InsP 5-ptase gene; rbcS 3′, 3′-untranslated region of the pea (*Pisum sativum*) rbcS E-9 gene; X, *Xba*I restriction enzyme sites; Km^R, kanamycin resistance cassette; black arrows, T-DNA border sequences. B, RNA gel-blot analysis. RNA was isolated from transgenic and wild-type cell lines harvested on d 4 of the culture cycle. Total RNA (5 μg lane^{-1}) was electrophoresed on a formaldehyde agarose gel and transferred to a nylon membrane. The blot was probed with ^{32}P-labeled InsP 5-ptase cDNA probe (upper) and stained with methylene blue to visualize RNA loading (lower). The arrow points to an approximately 1.7-kb transcript corresponding to InsP 5-ptase. WT, Wild type; C-3 and C-5, vector controls; I2-2, I2-8, and I2-11, transgenic lines transformed with InsP 5-ptase. C, Western blot of cell lysates. Cell lysates from transformed and wild-type tobacco cells were separated by SDS-PAGE electrophoresis (20 μg protein lane^{-1}), transferred to polyvinylidene difluoride (PVDF) membrane, and immunostained using antisera raised against the His tag. The antiserum recognizes a protein of approximately 45 kD that is detectable only in cells transformed with the InsP 5-ptase construct. The size of the protein expressed in tobacco cells is comparable with the purified recombinant InsP 5-ptase protein produced in bacteria pQE31 (<0.5 μg lane^{-1}). The lower panel shows the same blot stained with Amido black to visualize total protein.

vector control cell lines. Because the mammalian type I InsP 5-ptases share little overall homology (approximately 10%) with putative InsP 5-ptase genes from plants, it is not surprising that under the hybridization conditions used, no endogenous plant InsP 5-ptase transcripts were detected in the tobacco cells.

To establish whether the heterologous gene product was correctly synthesized in the tobacco cells, protein extracts were tested for the presence of InsP 5-ptase protein. Consistent with the expression of InsP 5-ptase transcripts described above, InsP 5-ptase protein could be detected in lines transformed with the InsP 5-ptase gene (I2-2, I2-8, and I2-11), but not in wild-type cells or lines transformed with the binary vector alone (C-3 and C-5). A western blot of protein extracts prepared from 4-d-old tobacco cell cultures, incubated with antiserum recognizing the His tag (Qiagen, Valencia, CA), is shown in Figure 1C. The antiserum reacted with a polypeptide of 45 kD, present only in the InsP 5-ptase cell lines (I2-2, I2-8, and I2-11) and not in wild-type or vector control (C-3 and C-5) tobacco lines. The size of the protein (approximately 45 kD) detected in the NT1 cells is identical to that of the active recombinant InsP 5-ptase protein produced in a bacterial overexpression system (pQE31), suggesting that the InsP 5-ptase gene is correctly translated in the tobacco cells.

InsP 5-ptase Expression Does Not Affect Cell Growth or Overall Cell Morphology

To determine whether InsP 5-ptase transgene expression had adverse effects on the transformed tobacco cells, cell growth, viability, and morphology were monitored over the culture cycle. Figure 2A illustrates the growth of wild-type, vector control, and InsP 5-ptase lines over a 7-d culture period. Upon transfer to fresh media, the tobacco cells typically exhibited a 2-d lag period followed by a period of exponential growth between d 3 and 6 of culture. As seen in Figure 2A, there were no significant differences in the growth rates of the wild-type and transformed cell lines over the culture cycle. Between d 6 and 7 of the culture cycle, the InsP 5-ptase cell line I2-8 showed a slight reduction in growth compared with the wild type. The growth curve of the other InsP 5-ptase line I2-2 was very similar to that of the wild-type culture.

Cell morphology and viability were examined microscopically on d 2, 4, and 6 of the culture cycle. Figure 2B shows cells from d 4 of the culture cycle stained with the vital stain, fluorescein diacetate. The

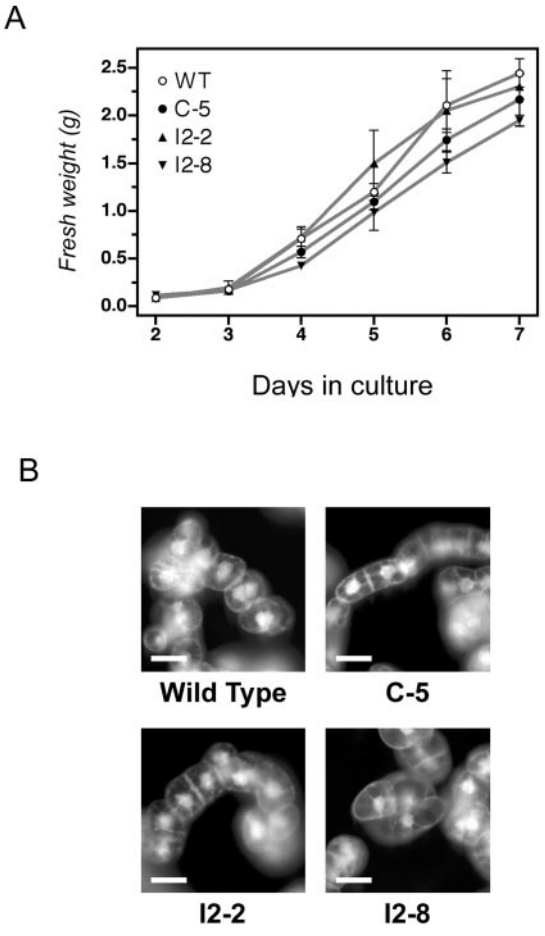

Figure 2. Cell growth and morphology of wild-type and transgenic tobacco cells. A, Cell samples from 5-mL cultures were harvested daily over the culture cycle and the fresh weight monitored. Three replicate cultures of each sample were harvested at each time point. A representative growth curve (drawn through the average values for each time point) from one experiment is shown in the figure. The bars show the range in fresh weight values obtained from the replicate cultures at each time point. The experiment was repeated twice with similar results. B, Fluorescence microscopy of cells from d 4 of the culture cycle. Cells were stained with 0.01% (w/v) fluorescein diacetate in acetone. The scale bar represents 25 μm.

overall morphology of the InsP 5-ptase lines as observed by light microscopy was not altered compared with the wild type over the culture period. Cell viability, monitored at d 2, 4, and 6 of the culture cycle, was found to be uniformly high in all of the cell lines (>90%). All subsequent experiments were conducted with cells from d 4 of the culture cycle (early exponential phase). Care was taken to perform experiments with cells exhibiting similar growth rates (6–7 g fresh weight of 50 mL^{-1} of culture at d 4).

Increased InsP 5-ptase Enzyme Activity in Transgenic Tobacco Cells Results in Reduced Basal $InsP_3$ Levels

To determine whether the InsP 5-ptase protein produced in the transgenic lines was catalytically active, the $InsP_3$-hydrolyzing activity of lysates from tobacco cells transformed with the InsP 5-ptase was compared with that of lysates from wild-type and vector control cells. Protein lysates (separated into microsomal and soluble fractions) prepared from 4-d-old cells were incubated for 10 min with commercially available $InsP_3$. After incubation, the $InsP_3$ content in the samples was measured. After 10 min of incubation, only approximately 1% of the total $InsP_3$ was hydrolyzed by fractions from wild-type and vector control lines, signifying low endogenous InsP 5-ptase activity (Fig. 3A). The InsP 5-ptase activity in both microsomal and soluble fractions of wild-type and vector control lines ranged from approximately 4.2 to 6.4 pmol min^{-1} mg^{-1} protein. Under the same conditions, InsP 5-ptase activity in extracts from the transgenic cell lines was increased 12- to 25-fold compared with the wild-type and vector control lines. The specific activity of InsP 5-ptase was higher in the microsomal fractions (approximately 106 pmol min^{-1} mg^{-1} protein) of the InsP 5-ptase lines compared with the soluble fractions (approximately 80 pmol min^{-1} mg^{-1} protein). The calculated specific activity values may be an underestimation because approximately 80% of the commercial $InsP_3$ was hydrolyzed by the transgenic microsomal fractions and the reactions may have saturated by 10 min. No significant differences in the endogenous InsP 5-ptase activity between microsomal and soluble fractions were detected for both the wild-type and vector control lines.

The increase in InsP 5-ptase activity in the microsomal fractions of the transgenic tobacco cell lines maybe due to the fact that the mammalian type I InsP 5-ptase contains a C terminal isoprenylation site (CAAX), which is thought to be important for membrane association. In animal cells, the enzyme is found in both the cytosolic and particulate fractions. Deletion or mutation of the Cys residue in the putative isoprenylation site of the mammalian InsP 5-ptase results in a predominantly cytosolic localization (De Smedt et al., 1996). Because isoprenylation motifs are conserved between plants and animals (Randall and Crowell, 1999) it is likely that the transgene product shows a similar subcellular distribution in the tobacco cells as in mammalian cells.

To determine whether the increased InsP 5-ptase activity affected the endogenous levels of $InsP_3$ in the transgenic cell lines in vivo, $InsP_3$ levels were measured in wild-type, vector control, and InsP 5-ptase lines at d 4 of the culture cycle. As seen in Figure 3B, both wild type and the vector control line (C-5) exhibited similar levels of $InsP_3$ (150–200 pmol g^{-1} fresh weight), whereas the three InsP 5-ptase lines (I2-2, I2-8, and I2-11) showed a 60% to 80% reduction in the levels of endogenous $InsP_3$ (20–60 pmol g^{-1} fresh weight). Reductions in basal $InsP_3$ levels of the InsP 5-ptase lines compared with the wild type and vector control were also observed at d 2 and 6 of the culture cycle (data not shown), indicating that the

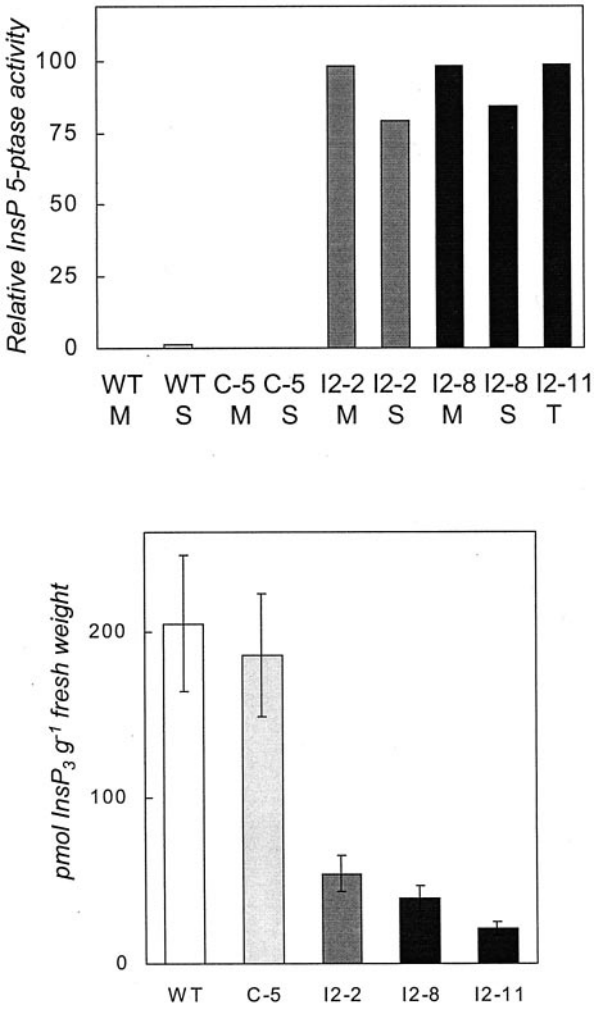

Figure 3. Increased activity of the InsP 5-ptase protein and decreased basal $InsP_3$ levels in the transgenic tobacco cells. A, InsP 5-ptase activity of cell lysates. Microsomal (M) or soluble (S) protein fractions (30 μg protein assay^{-1}) from transformed and wild-type tobacco cells were incubated for 10 min at room temperature with commercial $InsP_3$. Relative InsP 5-ptase activity was plotted as the percentage of $InsP_3$ hydrolyzed by each fraction compared with the wild-type control. The data are the averages of three independent experiments assayed in duplicate. The variation between experiments is <5%. Sample I2-11 was only assayed as a total protein extract. B, $InsP_3$ levels of wild-type and transformed cells from d 4 of the culture cycle were measured as described in "Materials and Methods." Data plotted are the average of four independent experiments assayed in duplicate.

ectopically expressed InsP 5-ptase was active throughout the culture cycle.

Expression of InsP 5-ptase in Transgenic Tobacco Cells Attenuates $InsP_3$ Changes in Response to Mastoparan Treatment

To determine whether expression of the InsP 5-ptase would impair the ability of the transgenic tobacco cells to generate $InsP_3$ signals upon stimulation, wild-type cells and the transgenic line I2-8 were treated with a stimulus known to activate PLC and increase $InsP_3$ levels in plant cells. Treatment with mastoparan, or its synthetic analog Mas-7, has been shown to increase $InsP_3$ levels rapidly and transiently in suspension-cultured carrot (*Daucus carota*) and soybean (*Glycine max*) cells (Legendre et al., 1993; Drøbak and Watkins, 1994; Cho et al., 1995) and in the green algae *Chlamydomonas reinhardtii* and *Chlamydomonas moewusii* (Quarmby et al., 1992; Munnik et al., 1998b). Application of mastoparan (in the range of 1–25 μM) resulted in a concentration-dependent increase in $InsP_3$ levels with an approximately 4- to 5-fold increase in $InsP_3$ at 25 μM (Legendre et al., 1993; Drøbak and Watkins, 1994). Both mastoparan and its synthetic analog Mas-7 have been shown to induce Ca^{2+} influx into animal (Suh et al., 1996) and plant cells (Franklin-Tong et al., 1996; Tucker and Boss, 1996; Takahashi et al., 1998); however, the exact mechanism of action of mastoparan in plant cells is not known.

Wild-type and InsP 5-ptase-expressing cells (I2-8) were treated with 5 μM Mas-7 or the inactive analog Mas-17, and $InsP_3$ levels were measured over the first few minutes. Within 1 min of treatment, there was a rapid, transient, 2- to 3-fold increase in $InsP_3$ in the wild-type cells, consistent with the previous reports from carrot and soybean cells (Fig. 4). No changes in $InsP_3$ were detected in response to treatment with the

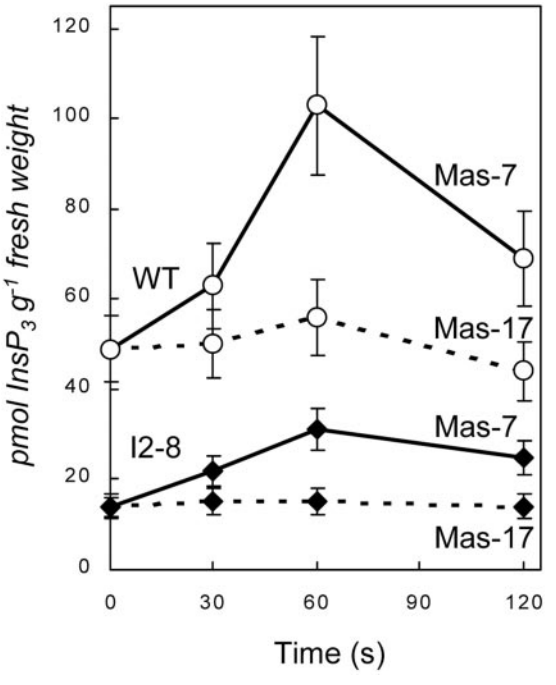

Figure 4. $InsP_3$ changes in response to mastoparan treatment. Changes in $InsP_3$ levels in response to 5 μM Mas-7 (solid lines) or 5 μM Mas-17 (dotted lines) in 4-d-old wild-type cultures (white circles) and InsP 5-ptase-expressing cell line I2-8 (black diamonds) over the first few minutes after stimulation. The data plotted are the average of four independent experiments assayed in duplicate and the error bars show the range.

inactive analog Mas-17. The InsP 5-ptase expressing line I2-8 also showed an approximately 2-fold increase in $InsP_3$ over basal levels at 1 min after treatment with 5 μM Mas-7. However, even after stimulation, the levels of $InsP_3$ in the transgenic cells did not reach the wild-type basal levels and the transient increase in $InsP_3$ with Mas-7 treatment was greatly attenuated in cells expressing the InsP 5-ptase.

Expression of InsP 5-ptase in Transgenic Tobacco Cells Affects Upstream PI Metabolism

To determine whether the reduction in basal $InsP_3$ levels in the transgenic cell lines had an impact on the levels of $PtdInsP_2$ (the lipid precursor of $InsP_3$), total cellular $PtdInsP_2$ levels were measured. Figure 5A shows that the wild-type and vector control cell lines contained comparable amounts of $PtdInsP_2$, based on mass measurements. In contrast, there was an approximately 70% reduction in $PtdInsP_2$ in cells expressing the InsP 5-ptase. These data suggest that the increased hydrolysis of $InsP_3$ had a direct effect on PI metabolism. We also examined the levels of inositol phospholipids by labeling cells in vivo with [^{3}H] inositol (Table I). After 24 h of labeling, there was a small but reproducible reduction in the levels of [^{3}H] $PtdInsP_2$ and [^{3}H] PtdInsP in the transgenic lines compared with the wild type. Although it is more difficult to assess total lipids by in vivo labeling, these results are consistent with the reduction in whole-cell $PtdInsP_2$ determined by mass measurements.

The decrease in whole-cell $PtdInsP_2$ could have resulted from decreased synthesis or increased flux through the PI pathway. To investigate the potential effects of the transgene expression on $PtdInsP_2$ biosynthesis, the specific activity of PtdInsP 5 kinase (the enzyme responsible for phosphorylating PtdInsP to $PtdInsP_2$) was measured in both microsomes and plasma membrane-enriched fractions isolated by two-phase partitioning from wild-type, vector control, and InsP 5-ptase-expressing lines. Membrane fractions were incubated with [γ-^{32}P] ATP and excess lipid substrate, PtdIns4P. Lipids were extracted and separated by TLC and the radiolabeled lipid products analyzed with an imaging scanner (Bioscan, Washington, DC). The specific PtdInsP 5 kinase activity in microsomes from all of the transformed lines including the vector control increased slightly (30%–50%) compared with the wild type. In contrast, the specific PtdInsP 5 kinase activity in plasma membranes of tobacco cells expressing InsP 5-ptase increased approximately 3-fold compared with the wild type and vector control line (Fig. 5B). These results show that although the total amount of $PtdInsP_2$ was reduced, the ability of the plasma membranes to synthesize $PtdInsP_2$ had increased in the transgenic cell lines.

We also investigated the possibility that the plasma membrane PI-PLC activity might be increased in the transgenic lines to compensate for the increased $InsP_3$ hydrolysis. As seen in Figure 5B, the plasma membrane PLC activity only increased approximately 15% to 20% in the InsP 5-ptase lines, suggesting that although the rate of $InsP_3$ hydrolysis had increased, PLC activity was not significantly affected.

To determine whether increasing InsP 5-ptase activity resulted in an increased flux through the PI pathway, the rate of $PtdInsP_2$ biosynthesis was measured in vivo. ^{32}Pi was added to transgenic and wild-type tobacco cells on d 4, equivalent aliquots of cells were harvested at each time point, and the lipids were extracted and separated by TLC. As seen in Figure 5C, the incorporation of ^{32}P into $PtdInsP_2$ over the first 10 min was 4- to 5-fold higher in the transgenic line compared with the wild type, consistent with an increase in $PtdInsP_2$ biosynthesis in the transgenic cells.

DISCUSSION

In eukaryotic cells, the intermediates of the PI pathway are maintained by the concerted action of multiple lipid kinases, lipases, and phosphatases. The membrane-associated phospholipid, PtdIns, is sequentially phosphorylated by specific lipid kinases (Drøbak et al., 1998) to form phosphatidylinositol 4-phosphate (PtdIns4P) and $PtdInsP_2$, the precursor of the second messenger, $InsP_3$. The sequential dephosphorylation of the membrane phospholipids and soluble inositol phosphates is brought about by specific inositol polyphosphate phosphatases (Zhang and Majerus, 1998). We have chosen to perturb the PI pathway in tobacco cells by the constitutive expression of the human type I InsP 5-ptase, which specifically hydrolyzes $InsP_3$.

The goals of this study were to: (a) generate stably transformed plant cultures expressing the heterologous gene, and (b) study the effects of constitutive expression of InsP 5-ptase on PI metabolism and $InsP_3$ production. We show that the human gene was expressed and active in the tobacco cells, and that the increased InsP 5-ptase activity resulted in a drastic reduction in the basal levels of $InsP_3$. The transformed cells showed no visible phenotype by light microscopy and the growth rates of the transgenic cell lines under normal growth conditions did not appear to be compromised by the expression of the transgene compared with wild-type cells. However, the transgenic lines exhibited a decrease in whole-cell $PtdInsP_2$ and an increased rate of $PtdInsP_2$ biosynthesis. These data suggest that constitutively increasing $InsP_3$ hydrolysis leads to an increased flux through the PI pathway.

The mammalian type I InsP 5-ptase is well characterized and specifically hydrolyzes the soluble inositol phosphates, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (Laxminarayan et al., 1993). Constitutive overexpression of the type I InsP 5-ptase in Chinese hamster ovary cells

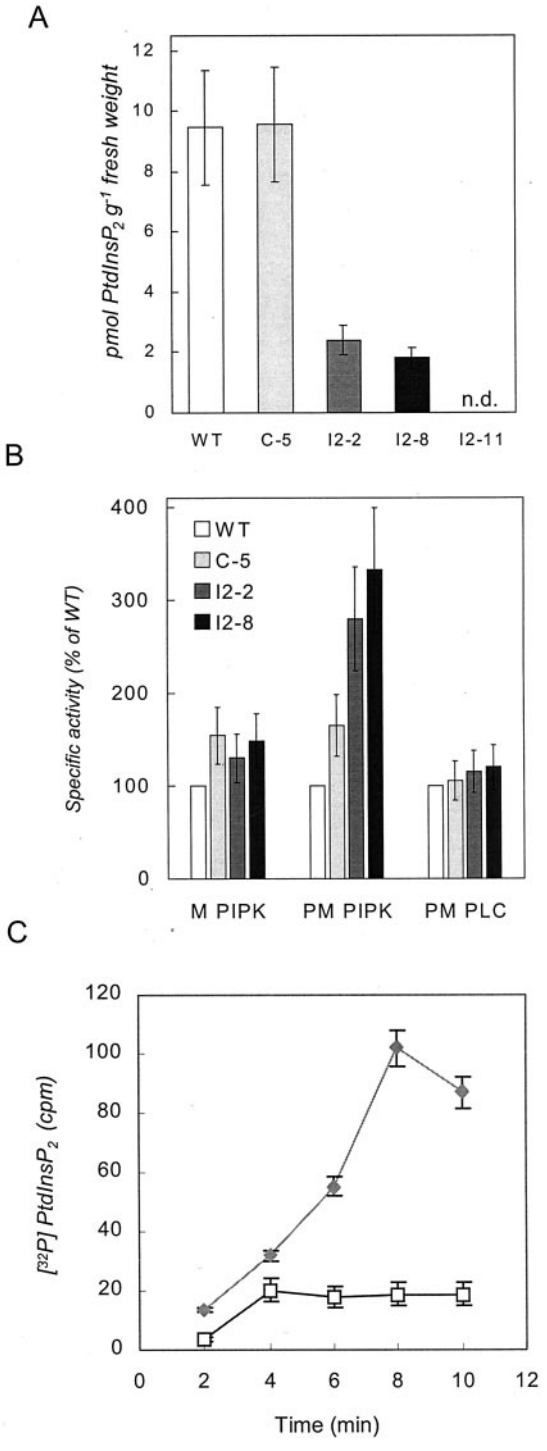

Figure 5. Altered PI metabolism in transgenic tobacco cells. A, $PtdInsP_2$ content of wild-type and transformed cells from d 4 of the culture cycle were measured as described in "Materials and Methods." Data plotted are the average of two independent experiments assayed in duplicate. B, Specific activity of PtdInsP 5 kinase from microsomes and plasma membranes and specific activity of PLC from plasma membranes at d 4 of the culture cycle. The specific activity of PtdInsP 5 kinase (PIPK) from microsomes (M) and plasma membrane-enriched fractions (PM) and the PLC activity from plasma membrane fractions of the transgenic tobacco cell lines was plotted as percentage of the wild type (set at 100%). Wild-type PtdInsP 5 kinase activity was typically 12 and 180 pmol min^{-1} mg^{-1} for microsomes and plasma membranes, respectively. PLC activity of plasma membranes from wild type was 1 to 2 nmol min^{-1} mg^{-1} membrane protein. The samples were assayed in duplicate and the values plotted are the average of three independent experiments. The error bars show the range. C, Incorporation of ^{32}Pi into $PtdInsP_2$ in wild-type (open squares) and transgenic (closed diamonds) tobacco cells over a 10-min labeling period. Equivalent cell samples were removed at the specific time points and the lipids were extracted, separated by thin-layer chromatography (TLC), and quantified as described in "Materials and Methods." Data plotted are the average of duplicates from a representative experiment. Similar results were obtained in two independent experiments.

Table I. *Distribution of [^{3}H] inositol lipids in wild-type and transgenic tobacco cells*

The values for [^{3}H] PtdIns, [^{3}H] PtdInsP, and [^{3}H] $PtdInsP_2$ are given as a percentage of the total [^{3}H] inositol-labeled lipids recovered and are the averages ± SD from four independent experiments assayed in duplicate. (The radioactivity recovered as total [^{3}H] inositol-labeled lipids was routinely ~40,000 cpm).

	[^{3}H] Inositol-Labeled Lipids		
	PtdIns	PtdInsP	$PtdInsP_2$
		%	
Wild type	80 ± 6	11.4 ± 2	1.32 ± 0.2
I2-2	79 ± 7	9.9 ± 0.4	0.60 ± 0.2
I2-8	82 ± 4	8.6 ± 1	0.85 ± 0.2

(CHO-K1) resulted in a 15- to 25-fold increase in InsP 5-ptase activity and approximately 75% reduction in $InsP_3$ levels (De Smedt et al., 1997). Consistent with the results from animal cells, in this study, the tobacco cells constitutively expressing the human InsP 5-ptase showed a 12.5- and 25-fold increase in $InsP_3$-hydrolyzing activity in soluble and microsomal fractions, respectively, and basal $InsP_3$ levels were reduced by 60% to 80% in the transgenic cell lines compared with wild type and vector controls.

In CHO-K1 cells, overexpression of the type I InsP 5-ptase significantly affected $InsP_3$-mediated Ca^{2+} responses to both high and low concentrations of agonist (De Smedt et al., 1997). In the transformed CHO-K1 cells, the rapid clearing of the agonist-induced $InsP_3$ by the InsP 5-ptase dampened the activation of $InsP_3$ receptors and the generation of Ca^{2+} oscillations (De Smedt et al., 1997). Although we have not examined responses downstream of $InsP_3$ in the transgenic tobacco cells expressing InsP 5-ptase, we show that production of $InsP_3$ with Mas-7 treatment was attenuated in these cells and that even after stimulation, $InsP_3$ levels remained below wild-type basal levels. With Mas-7 treatment, there was a transient increase in $InsP_3$ in the transgenic cells, suggesting that activation of PLC was not impaired. However, the magnitude of the $InsP_3$ increase was severely attenuated, consistent with the reduction in basal $InsP_3$. This reduced capacity to generate $InsP_3$ could have a great impact on the cells' ability to respond to a stimulus.

The increased rate of $InsP_3$ hydrolysis in the transgenic tobacco cells could have been compensated for by an up-regulation of $PtdInsP_2$ hydrolysis. Surprisingly, we detected only minor (15%–20%) increases in PLC activity of plasma membrane-enriched fractions from the InsP 5-ptase transgenic cell lines. In contrast to the modest increase in plasma membrane PLC activity, the specific activity of the plasma membrane PtdInsP 5 kinase from transformed tobacco cultures increased approximately 3-fold in vitro. An increased rate of $PtdInsP_2$ synthesis was confirmed by in vivo labeling studies. These results indicate that the plasma membrane PtdInsP 5 kinase was activated as a compensatory mechanism for the increased demand for $PtdInsP_2$ resulting from increased $InsP_3$ hydrolysis.

$PtdInsP_2$ is essential for the regulation of many cellular processes including cytoskeletal dynamics, vesicle trafficking, and ion transport and enzyme activity (for discussion, see Janmey, 1994, 1998; Toker, 1998; Stevenson et al., 2000). It is evident that there are dynamic, functionally distinct pools of $PtdInsP_2$ within the cell that vary with the physiological state of the cell (Hinchliffe et al., 1998; Heilmann et al., 2001), and $PtdInsP_2$ is emerging as a signaling molecule in its own right (Czech, 2000; Hinchliffe, 2000; Raucher et al., 2000). The drastic decrease in whole-cell $PtdInsP_2$ in the transgenic cells (approximately 70% reduction compared with wild type) could, therefore, significantly affect global cellular function; however, no morphological differences could be detected by light microscopy between the transgenic and wild-type tobacco cells. The viability of the transgenic cells in culture was also comparable with that of the wild-type cells. These observations suggest that under the favorable growth conditions of cell culture, the transgenic cells have adjusted to a new steady-state level with lowered PIs and that morphological changes may more likely be manifested under limiting conditions or at different developmental stages in a whole-plant system.

All enzymes in a metabolic pathway can potentially contribute toward the control of flux (Thomas and Fell, 1998). In eukaryotic cells, the steady-state levels of $PtdInsP_2$ in the plasma membrane pools are maintained by the activity of several enzymes involved in $PtdInsP_2$ turnover. $PtdInsP_2$ biosynthesis is dependent on the availability of the precursor lipid PtdInsP as well as on the activity of PtdInsP 5 kinase. Catabolism of $PtdInsP_2$ may occur either via dephosphorylation by inositol polyphosphate phosphatases or hydrolysis by PLC.

Association with cytoskeletal elements and other lipid-binding proteins can limit the availability of the lipids PtdInsP and $PtdInsP_2$ for enzyme action (Drøbak et al., 1994; Toker, 1998; Stevenson et al., 2000). The availability of lipid substrates may also be controlled by the lipid transfer proteins (PITPs). In animal cells, PITP-mediated delivery of the precursor lipids to the plasma membrane for $PtdInsP_2$ synthesis is highly regulated (Cunningham et al., 1995; Speed and Mitchell, 2000). Plant PITP-like proteins are biochemically distinct from their animal counterparts and may serve specialized functions (Drøbak et al., 1998; Kapranov et al., 2001). Because the ratio of PtdInsP:$PtdInsP_2$ in plants is on the order of 10:1, it is unlikely that PtdInsP is a limiting factor for $PtdInsP_2$ biosynthesis (Stevenson et al., 2000).

In animal cells, regulation of $PtdInsP_2$ biosynthesis also occurs via activation or translocation of PtdInsP 5 kinase enzymes (for review, see Hinchliffe et al., 1998). Similarly, in plants, regulation of PtdInsP 5 kinase activity by phosphorylation (Westergren et al., 2001) and translocation of the enzyme (I. Heilmann and W.F. Boss, unpublished data) could contribute toward the control of $PtdInsP_2$ biosynthesis. Recent evidence shows that PtdInsP 5 kinase activity and $PtdInsP_2$ synthesis in plants change in response to stress. An up-regulation of PtdInsP 5 kinase activity was observed in maize (*Zea mays*) pulvini upon gravistimulation (Perera et al., 1999), and in *Galdieria sulphuraria* cells in response to hyperosmotic stress (Heilmann et al., 1999, 2001). Furthermore, in *G. sulphuraria* cells with low levels of plasma membrane $PtdInsP_2$, the increase in PtdInsP 5 kinase activity preceded $InsP_3$ production, suggesting that PtdInsP 5 kinase activity is highly sensitive to the demands of $PtdInsP_2$ turnover. Other groups have also documented increases in $PtdInsP_2$ biosynthesis with hyperosmotic stress (Pical et al., 1999; DeWald et al., 2001; Takahashi et al., 2001) and with mastoparan treatment (Munnik et al., 1998b), implicating the activation of the PtdInsP 5 kinases. There is also evidence that PtdInsP 5 kinase gene expression increases with salt and drought stress in Arabidopsis (Mikami et al., 1998). These findings support the importance of PtdInsP 5 kinase in the regulation of $PtdInsP_2$ levels.

The mechanisms involved in $PtdInsP_2$ catabolism in plants are less well understood. In particular, the inositol polyphosphate phosphatases of plants that target phospholipids have not been studied in detail (Drøbak, 1992). Information on the in vivo regulation of plant PLC enzymes is also limited. Although animal PLC enzymes comprise a large group of four families (Rhee, 2001), which can be activated via different effectors, all the plant PLC genes characterized to date are most similar to the animal PLC δ family. Furthermore, the plant enzymes lack the regulatory PH domain. The PH domain of the mammalian PLC-δ1 has been shown to specifically interact with $PtdInsP_2$ and $InsP_3$ and may be involved in the translocation of the enzyme to and from the membrane (Cifuentes et al., 1994). Plant $PtdInsP_2$-hydrolyzing PLC activity is primarily associated with membrane fractions and Ca^{2+} is required for activation (Hirayama et al., 1995; Otterhag et al., 2001). Both constitutive, and at least at the level of

transcript, inducible, isoforms of PLC have been described in Arabidopsis (Hirayama et al., 1995, 1997), supporting the idea that PLC activity increases in response to stimulation. However, increased expression of AtPLC1 did not lead to an increase in $InsP_3$ levels in transgenic plants (Sanchez and Chua, 2001). Taken collectively, these observations suggest that the synthesis of $PtdInsP_2$ is a major factor influencing $PtdInsP_2$ homeostasis, which in turn is a critical determinant for $InsP_3$ production in plants.

The results of our study suggest that the constitutive expression of a heterologous and highly active InsP 5-ptase in tobacco cells has created a "drain" for $InsP_3$. The metabolic consequence of the elevated $InsP_3$ hydrolysis could be that the transgenic cells are in a continuous state of attempting to replenish a pool of $PtdInsP_2$ through activation of the plasma membrane PtdInsP 5 kinase. Further work will be needed to characterize the altered pools of $PtdInsP_2$ at a subcellular level and evaluate the impact of these changes on downstream physiology.

MATERIALS AND METHODS

Plant Materials

Tobacco (*Nicotiana tabacum*) tissue culture cells (NT1 cells) were maintained in 50 mL of liquid culture medium (1× Murashige and Skoog salts [Gibco BRL, Bethesda, MD], 0.18 g L^{-1} KH_2PO_4, 0.1 g L^{-1} myo-inositol, 1 mg L^{-1} thiamine HCl, 0.2 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 30 g L^{-1} Suc [pH 5.7]) at 28°C with shaking at 125 rpm, in darkness. Cells were subcultured weekly with a 6% (v/v) inoculum.

Monitoring Cell Growth and Microscopy

For monitoring cell growth over the culture cycle, three replicate 5-mL cultures were harvested daily and the fresh weight was recorded. At 2, 4, and 6 d after transfer, cell samples were stained with 0.01% (w/v) fluorescein diacetate in acetone (Widholm, 1972) and cell viability was determined by fluorescence microscopy, using a Axiovert 100 TV microscope (Zeiss, Jena, Germany) coupled to a cooled CCD camera. Images were resolved using a 25× 2EIBS plan-neofluar lens. Fluorescence excitation was by a super-high pressure mercury lamp in front of a 470- ± 40-nm barrier filter. Fluorescence emission was filtered using a 510-nm dichroic mirror and a 540- ± 50-nm filter. Bright-field images were also recorded for each field of view.

Plant Transformation and Selection of Transgenic Lines

The cDNA encoding the human type I InsP 5-ptase (accession no. X77567) was subcloned into the *Xba*I site of the pKYL71-35S^2 binary vector (Schardl et al., 1987). The pKYL71-35S^2 vector contains a modified 35S promoter, 3′-untranslated region of the pea (*Pisum sativum*) rbcS E-9 gene, and a plant kanamycin resistance cassette. The coding region of the InsP 5-ptase gene, along with the 5′ end His tag, was amplified from the bacterial expression vector pQE31 using forward and reverse primers engineered to contain *Xba*I sites. The orientation of the resulting plasmid, pKYL71-35S^2-InsP 5-ptase, was verified by restriction enzyme analysis and DNA sequencing.

NT1 cells were transformed using *Agrobacterium tumefaciens*-mediated gene transfer following the protocol of Persson et al. (2001). The binary plasmids, pKYL71-35S^2-InsP 5-ptase and pKYL71-35S^2 (vector control), were electroporated into *A. tumefaciens* strain LBA-4404, using a Gene Pulser system (Bio-Rad, Hercules, CA). A single-transformant *A. tumefaciens* colony for each plasmid was cultured in 5 mL of yeast extract broth media (0.5% [w/v] beef extract, 0.5% [w/v] peptone, 0.5% [w/v] Suc, 0.1% [w/v] yeast extract, and 2.5 mM $MgCl_2$) containing 50 mg L^{-1} kanamycin, at 27°C, with shaking at 250 rpm for 2 d. Wild-type NT1 cells were cultured for 4 d in 50 mL of NT1 culture medium, at 28°C, with shaking at 125 rpm. Four milliliters of this culture was gently mixed with 200 μL of 2-d-old *A. tumefaciens* cultures transformed with either pKYL71-35S^2-InsP 5-ptase or vector control pKYL71-35S^2. The NT1 cell-*A. tumefaciens* mix was incubated for 48 h at 28°C and suspended in an equal volume of NT1 culture medium. Approximately 0.5 mL of the resulting cell suspension was plated onto solid NT1 culture medium containing 0.8% (w/v) Phytagar (Gibco BRL), 50 μg mL^{-1} kanamycin, and 200 μg mL^{-1} timetin, and excess liquid allowed to dry. Plates were incubated for 14 d at 28°C. For each transformation, 15 independent, kanamycin-resistant microcalli were picked, suspended in 1 mL of NT1 medium containing 50 μg mL^{-1} kanamycin and 200 μg mL^{-1} timetin, and incubated for 7 d at 28°C, at 190 rpm, in darkness. Suspension cultures that formed under these conditions were retained and transferred to 4 mL of NT1 culture medium containing 50 μg mL^{-1} kanamycin and 200 μg mL^{-1} timetin, and incubated for 7 d as described above. To maintain the lines, cells were subcultured weekly, as described above, in 5 mL of NT1 culture medium containing 50 μg mL^{-1} kanamycin.

To verify transformation and determine if the transgene was expressed, independently, transformed NT1 cell lines were harvested after 4 d of growth and frozen in liquid N_2. One hundred milligrams of frozen cells was ground and extracted in 100 μL of 2× SDS-PAGE sample buffer and the crude lysates were separated by SDS-PAGE. After electrophoresis proteins were electroblotted onto hydrophobic PVDF membranes (Gelman Sciences, Ann Arbor MI) and the blots were probed with a monoclonal antiserum that recognizes the His tag (RGS-His monoclonal antibody, Qiagen). Of 15 transformed lines, 11 showed InsP 5-ptase expression, whereas no protein was detected in wild type and vector-only controls. Three of the cultures that showed high InsP 5-ptase expression were selected for further analysis and maintained weekly by subculturing in 50 mL of NT1 culture medium containing 50 μg mL^{-1} kanamycin.

RNA Extraction and RNA Gel-Blot Analysis

RNA was isolated from 4-d old-NT1 cultures (approximately 0.2 g fresh weight) using a plant RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA (5 μg) was electrophoresed on a 1% (w/v) agarose gel in formaldehyde (Perera and Zielinski, 1992) and transferred to Magna-Graph nylon transfer membrane (Osmonics Lab Products, Minnetonka, MN) overnight in 20× SSPE (0.2 M NaH_2PO_4·water/NaOH [pH 7.4], 20 mM $Na_2EDTA{\cdot}2H_2O$, and 2.98 M NaCl). Blots were prehybridized for 3 to 4 h at 44°C and hybridized at the same temperature for 16 h. Prehybridization and hybridization was carried out in 50% (v/v) formamide, 5× SSPE, 5× Denhardt's solution (100× Denhardt's solution is 2% [w/v] each of bovine serum albumin [BSA], polyvinylpyrrolidone, and Ficoll 400), 100 μg mL^{-1} denatured calf thymus DNA, and 0.5% (w/v) SDS. The probe was prepared by oligolabeling of the InsP 5-ptase cDNA with α-[^{32}P] dCTP and random hexamer primers using DNA polymerase Klenow fragment (Promega, Madison, WI) were washed twice at room temperature in 2× SSPE/0.2% (w/v) SDS, followed by washes in 1× SSPE/0.1% (w/v) SDS at room temperature and hybridization temperature. The final washes were in 0.1× SSPE/0.1% (w/v) SDS at 44°C and 55°C. Hybridization was visualized by autoradiography.

Preparation of Total Protein Lysates, Microsomes, and Plasma Membranes

NT1 cells were harvested at d 4 by filtration and immediately homogenized in 3 volumes of cold buffer (200 mM Suc, 30 mM Tris/HCl [pH 7.2], 3 mM EGTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) in a glass Dounce homogenizer with 1% (w/v) polyvinylpolypyrrolidone to facilitate grinding. The crude extract was clarified by centrifugation at 5,000*g* for 10 min at 4°C. The supernatant was used as total cell lysate or fractionated further (40,000*g*, for 60 min, at 4°C) to yield microsomal and soluble protein fractions. The microsomal pellet was washed in buffer (30 mM Tris/HCl [pH 7.2] and 15 mM $MgCl_2$), centrifuged at 40,000*g* for 30 min at 4°C, and the final pellet was resuspended in the same buffer. Plasma membrane-enriched fractions were prepared from microsomes by aqueous two-phase partitioning as described previously (Perera et al., 1999). For enzyme assays, membrane fractions were placed on

ice and assayed immediately. Protein concentrations were estimated using the Bio-Rad protein assay reagent with BSA as a standard.

Electrophoresis and Immunoblotting

Protein samples were boiled in SDS-PAGE sample buffer for 5 min, centrifuged briefly, and separated by electrophoresis by SDS-PAGE on 10% (w/v) polyacrylamide gels. For immunoblotting, proteins were transferred to PVDF membrane by electroblotting in 1× CAPS buffer containing 10% (v/v) MeOH for 1 h at 50 V. Membranes were blocked for 1 h in 3% (w/v) BSA in Tris-buffered saline buffer followed by two washes in Tris-buffered saline with 0.2% (v/v) Tween (TBST). Blots were incubated with the primary antibody (RGS-His; 1:1,500 [v/v] dilution in TBST) for 1 h followed by three washes in TBST. The blots were then incubated in the secondary antibody (horseradish peroxidase-conjugated anti-mouse; 1:20,000 [v/v] in TBST) for 1 h followed by three washes in TBST. Immunoreactivity was visualized by incubating the blot in SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) and exposure to x-ray film. After chemiluminescence detection, total protein was visualized by staining the blots with Amido black (Sigma, St. Louis).

Assay for InsP 5-ptase

Equal amounts of protein extracts (total lysate, microsomal, or soluble fractions) were incubated with commercial $InsP_3$ (30 pmol) in 100 μL of volume containing 3 mM $MgCl_2$ for 10 min at room temperature. Reactions were stopped by the addition of 5 mM EDTA. The $InsP_3$ remaining in each reaction after incubation was quantified using the assay described below. The amount of non-hydrolyzed $InsP_3$ remaining after incubation in the wild-type microsomal fraction was set to 100%. $InsP_3$ hydrolyzed by each fraction relative to the wild-type microsomal control (100-$InsP_3$ remaining in each sample) was plotted as a measure of InsP 5-ptase activity.

$InsP_3$ Assays and $PtdInsP_2$ Mass Measurements

Cells were harvested by filtration and immediately frozen in liquid N_2 and ground to a fine powder and precipitated with cold 20% (v/v) perchloric acid. For $InsP_3$ measurements, approximately 0.2 to 0.3 g (fresh weight) was used/assay. For the $PtdInsP_2$ measurements, 1.5 to 2 g (fresh weight) was used/assay. $InsP_3$ assays were carried out using the TRK1000 $InsP_3$ assay kit (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (Perera et al., 1999, 2001), and $PtdInsP_2$ mass measurements were carried out as described (Heilmann et al., 1999, 2001).

PtdInsP 5 Kinase Assays

In vitro lipid kinase assays were carried out using 30 μg of microsomal protein or 1 to 2 μg of plasma membrane protein. The standard assay contained 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM sodium molybdate, 50 μM γ[^{32}P] ATP (10 μCi reaction^{-1}), and 100 μM PtdIns4P (Avanti Polar Lipids, Alabaster, AL). Reactions were carried out at room temperature for 15 min in a total volume of 50 μL. After incubation, phospholipids were extracted by using an acidic $CHCl_3$/MeOH extraction method (Cho and Boss, 1995). Lipids were separated by TLC on LK5 silica gel plates (Whatman, Clifton, NJ) using a $CHCl_3$:MeOH:NH_4OH:water (90:90:7:22 [v/v]; Parker et al., 1998) solvent system. The ^{32}P-labeled phospholipids were quantified with a Bioscan System 500 Imaging scanner. Control reactions were carried out with purified bovine brain membrane (Sigma) to generate [^{32}P] $PtdInsP_2$ and in addition, [^{3}H]$PtdInsP_2$ (NEN, Boston) was used as a standard on the TLC plate.

PLC Assays

The PLC activity of plasma membrane fractions was monitored as described by Hirayama et al. (1995) with some modifications. Samples were incubated at room temperature for 10 min in buffer containing 50 mM Tris/HCl (pH 6.4), and 160 μM [^{3}H] PtdIns(4,5)P_2 (approximately 5,000 dpm nmol^{-1}), with 10 to 20 μM Ca^{2+} (as described by Cho et al., 1993) in a final volume of 50 μL. Reactions were stopped by adding 2 mL of cold $CHCl_3$:MeOH (2:1 [v/v]), followed by 0.5 mL of 1 N HCl. Samples were vortexed and centrifuged to separate phases and approximately 0.9 mL of the aqueous phase was removed and the radioactivity measured by liquid scintillation counting.

Mastoparan Stimulation

Synthetic peptides of mastoparan (Mas-7) and the inactive analog (Mas-17) were synthesized by the Microprotein Chemistry Facility of the Program of Molecular Biology and Biotechnology (University of North Carolina, Chapel Hill). Stock solutions (1.5 mM) of Mas-7 and Mas-17 were made up in water. Cell cultures (5 mL) were maintained on a shaker for the duration of the experiment. Mas-7 or Mas-17 were added to a final concentration of 5 μM and at the indicated times, samples were harvested by filtration and immediately frozen in liquid N_2. $InsP_3$ was analyzed as described above.

In Vivo Labeling of Cells

In vivo labeling was carried out with cells growing at the same rate with equivalent fresh weights. For 24-h labeling studies, 5 mL of cultures of 3-d-old wild-type and transgenic cells (approximately 0.1g cells mL^{-1}) were labeled with 20 μCi myo[2-^{3}H] inositol (20 Ci mmol^{-1}). After 24 h, cells were harvested by filtration, ground in liquid N_2, and incubated with cold 5% (v/v) perchloric acid containing 1 mg mL^{-1} phytate carrier for 15 min on ice. The pellet and supernatant were separated by centrifugation, the pellet was washed two times with cold water, and the lipids extracted as described previously (Cho and Boss, 1995). For short-term labeling, 4-d-old cells were harvested by filtration, weighed, and resuspended in conditioned medium (0.2g mL^{-1}). After a 30-min recovery period, cells were labeled with [^{32}P] Pi (70–80 μCi mL^{-1}). One-milliliter aliquots were removed at the indicated time points and added immediately to 1 mL of cold 20% (v/v) perchloric acid and incubated on ice for approximately 20 min. The pellet was washed two times with cold water and lipids were extracted, separated by TLC, and quantified as described above.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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