Phosphatidylinositol metabolism plays a central role in signaling pathways in animals and is also believed to be of importance in signal transduction in higher plants. We report here the molecular cloning of a cDNA encoding a previously unidentified 126-kDa phosphatidylinositol 4-kinase (AtPI4Kβ) from the higher plant Arabidopsis thaliana. The novel protein possesses the conserved domains present in animal and yeast PI 4-kinases, namely a lipid kinase unique domain and a catalytic domain. An additional domain, approximately 300 amino acids long, containing a high percentage (46%) of charged amino acids is specific to this plant enzyme. Recombinant AtPI4Kβ expressed in baculovirus-infected insect (Spodoptera frugiperda) cells phosphorylated phosphatidylinositol exclusively at the D4 position of the inositol ring. Recombinant protein was maximally activated by 0.6% Triton X-100 but was inhibited by adenosine with an IC50 of ~200 μM. Wortmannin at a concentration of ~10 μM inhibited AtPI4Kβ activity by ~90%. AtPI4Kβ transcript levels were similar in all tissues analyzed. Light or treatment with hormones or salts did not change AtPI4Kβ transcript levels to a great extent, indicating constitutive expression of the AtPI4Kβ gene.

Synthesis and hydrolysis of phosphoinositides play an important role in the transduction of physiological signals such as hormones, growth factors, and neurotransmitters in animal cells (1, 2). Sequential phosphorylation of the D4 and D5 positions of 1-α-phosphatidyl-1-D-myo-inositol (PtdIns) yields phosphatidylinositol 4-phosphate (PtdIns-4-P) and phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P2). PtdIns-4,5-P2 is hydrolyzed to inositol 1,4,5-trisphosphate, a stimulator of calcium release from intracellular stores (1), and diacylglycerol, an activator of some protein kinase C isoforms (3), by phosphoinositide-specific phospholipase C (PI-PLC). In addition to their classical function as precursors of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, phosphoinositides, including those phosphorylated at the D3 position of the inositol ring, have been shown to regulate cytoskeleton rearrangements through the association with a variety of actin-binding proteins, including profilin, gelsolin, and villin (4, 5), and can also, for example, potentiate the activation of protein kinase C (6) and PI-PLC (7, 8). They also constitute major regulators of membrane-trafficking (9, 10). With the exception of phosphatidylinositol 5-phosphate and phosphatidylinositol 3,4,5-trisphosphate, all of the inositol phospholipids found in animals have also been identified in plants. Focusing on the role of inositol lipids in plants, independent of their much questioned role in calcium signaling, PtdIns-4-P and PtdIns-4,5-P2 are known to affect the activity of several enzymes, including the plasma membrane H+/ATPase (11) and phospholipase D (12) and can interact, in vitro at least, with cytoskeletal components (13). Profilin is believed to be involved in cytoskeleton dynamics in plant cells as it is in animal cells, through interaction with PtdIns-4,5-P2 and actin (14).

PI 4-kinase catalyzes the phosphorylation of PtdIns to PtdIns-4-P, the first committed step toward the synthesis of PtdIns-4,5-P2, and therefore represents a potentially crucial point of regulation of the phosphatidylinositol-dependent pathways. Two types of PI 4-kinases, II and III, differing in size and sensitivity to detergents and adenosine, have been identified in a wide range of tissues and cellular compartments (15, 16). Type I PI kinases phosphorylate the D3 position of the inositol ring and are therefore referred to as PI 3-kinases (17).

cDNAs encoding functional PI 4-kinases have been isolated from animals and Saccharomyces cerevisiae. Putative PI 4-kinase clones have been identified in Schizosaccharomyces pombe, Dictyostelium discoideum (18), and Caenorhabditis elegans (19). The proteins encoded by these genes have now been grouped into two distinct subfamilies, 1.1 and 1.2, based on sequence and structure similarities (20). Subfamily 1.1 is represented by proteins of 68–122 kDa and subfamily 1.2 by proteins of 200–230 kDa (with the exception of human PI4Kα, which is a 97-kDa protein (21)). The comparison of the primary structures of these proteins has enabled the identification of several conserved domains. All PI 4-kinases possess a catalytic domain of about 230 amino acid residues, which constitute the C-terminal part of those proteins, and a so-called lipid kinase

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**A Plant 126-kDa Phosphatidylinositol 4-Kinase with a Novel Repeat Structure**

**CLONING AND FUNCTIONAL EXPRESSION IN BACULOVIRUS-INFECTED INSECT CELLS**

(Received for publication, November 16, 1998)

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ002685.

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The abbreviations used are: PtdIns, 1-α-phosphatidyl-1-D-myo-inositol; PtdIns-4-P, PtdIns 4-phosphate; PtdIns-4,5-P2, PtdIns 4,5-bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C; LKU, lipid kinase unique; PH, Pleckstrin homology; PCR, polymerase chain reaction; kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; bp, base pair(s).
regions. This fragment was subsequently used to screen an A. thaliana hypocotyl ZAP II cDNA library (CD4–16; obtained from the Arabidopsis Stock Center, Columbus, OH) under stringent conditions as previously described (33). Plaque-purified phage clones were converted to pBluescript derivatives using helper phage ExAssist according to the manufacturer’s (Stratagene) instructions. Clone pFBH-PI4Kn, which contained the longest cDNA insert, was used for further analyses.

DNA sequencing was done by MWG Biotech (Munich, Germany). Computational analysis was performed with the help of the programs of the Wisconsin Genetics Computer Group (GGC Package, Version 8.1 (34)). The FASTA (35) and BLAST (36) search programs were used for sequence comparisons on DNA and amino acid sequences in GenBank™, EMBL, dbEST, and SwissProt data bases. Sequence alignments were performed using the BESTFIT program. Short repetitive sequences within the AtPI4Kβ protein were identified with the help of the Saps program (37).

**Protein Expression in Insect Cells—Insect (Spodoptera frugiperda Sf21) cells (Invitrogen, Leek, The Netherlands) were cultivated as monolayer cultures as described (38) at 27 °C in TMN-FH medium (Sigma) supplemented with 10% fetal calf serum.**

**Expression of recombinant AtPI4Kβ protein, fused to a polyhistidine tag at its N terminus,** was achieved with the help of the Bac-to-Bac Expression System from Life Technologies, Inc. Transfer plasmid pFB-His-PI4K was constructed as follows. A DNA fragment encompassing an 820-bp region of the AtPI4Kβ cDNA was amplified via PCR using forward (5′-ATCGGGATCCTGCGATGAGTATTGTACGTTTA-3′) and reverse (5′-ATCCGGCGCGCCCTCAGTTGATATTGTTGTCAG-3′) primers and cloned into plasmid pFastBacHTb (Life Technologies, Inc.). The PCR-amplified fragment in the resulting plasmid pFBH-PI4K was sequenced to confirm that no nucleotide exchanges occurred during the PCR amplification. Subsequently, a 2.6-kb-long fragment of the AtPI4Kβ cDNA was inserted via NotI and NotI restriction sites into plasmid pFBH-PI4K. This final cloning step reconstituted the complete AtPI4Kβ-coding region. Recombinant baculoviruses obtained after transfection of insect cells with bacmid DNA (i.e. e. coli/baculovirus shuttle vector) were amplified twice to yield high titer virus stocks. For expression of recombinant protein, 9 ml of virus stock was used to infect cells (60–80% confluency) in 150 cm² culture flasks.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—**Proteins extracted from insect cells or obtained after protein purification (see below) were separated on 8% SDS-polyacrylamide gels (39). Western blot analysis was performed essentially as described previously (40). Proteins were transferred to the nitro blue tetrazolium/G-250-HiPhos (Ser-His-Glu-Glu-His) was used as a 1:1500 dilution in blocking buffer (0.3% lowfat milk powder (Heirler, Radolfzell, Germany), 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20). Alkaline phosphatase-conjugated secondary antibody (anti-mouse IgG; Promega, Madison, WI) was used at a 1:7500 dilution in TBST (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20, 0.1% bovine serum albumin). Blots were developed using nitro blue tetrazolium in conjunction with alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate.

**Biochemical Analysis of Recombinant AtPI4Kβ—**Recombinant AtPI4Kβ protein was purified from insect cells 3 days after infection. Cells were harvested, washed once with ice-cold phosphate-buffered saline buffer (10 mM Na2HPO4, 1.8 mM KH2PO4, 0.14 M NaCl, 2.7 mM KCl, pH 7.3), and lysed by sonication in lysis buffer containing 50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 10% glycerol, 20 μg/ml aprotinin, and 50 μg/ml chymostatin. Undisrupted cells were removed by centrifugation (14,000 rpm for 10 min, Centrifuge 5417, Eppendorf), and the supernatant was analyzed by SDS-PAGE or used for protein purification.

Recombinant protein was batch-purified under native conditions using nickel nitrotriacetic acid resin according to manufacturer’s (Qiagen) instructions. Yield of recombinant AtPI4Kβ from a representative purification (24 ml of insect cell culture) was 0.2 mg of protein. Purity of recombinant AtPI4Kβ was above 95% as estimated from Coomassie-stained SDS-PAGE protein gels. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard (41).

PI 4-kinase activity was determined at 37 °C in 50 μl of reaction buffer (50 mM Tris-HCl pH 7.4, 50 mM KCl, 10 mM MgCl2, 2 mM EDTA, 5 μg PtdIns (Sigma), 0.2% Triton X-100 (unless otherwise indicated), and 1 μg of affinity-purified protein. The reaction was started by the addition of 50 μl ω-32P[ATP (>600 Ci/mmol; ICN). The reaction was

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2 C. Fical, J. Kopka, F. Aitken, J.E. Gray, and B. Müller-Rober, unpublished information.
stopped after 30 min by adding 0.8 ml of cold chloroform/methanol/H2O (1/20/8; v/v/v). To extract lipids, 0.4 ml of chloroform/2.4 n HCl (1/1; v/v) was added, and the reaction mixture was vortexed and spun for 30 s at 12,000 rpm. The upper phase was removed, and an equal volume of chloroform/methanol (5/24/5; v/v/v) was used to wash the chloroform phase a second time. An aliquot of the chloroform phase (15 μl) was applied to thin-layer chromatography (TLC) plates (Silica gel 60 plates; Merck). Chromatograms were developed with a chloroform/methanol/concentrated NH4H2O (45/35/2/8, v/v/v) solvent mixture. Plates were exposed to Kodak X-Omat AR films. TLC revealed that 32P was present in a P1 clone (MHJ24; GenBank accession number AF012589). The calculated molecular weight of the gene had a size of approximately 8 kb, including 5′ and 3′ untranslated regions and putative 5′ regulatory elements. Mapping analysis indicated that the AtPI4Kβ gene is located at the bottom of chromosome V, between markers CIC9B5L and T04492.

**Structural Organization of the A. thaliana PI 4-Kinase Protein**—The deduced AtPI4Kβ protein sequence possessed two regions highly similar to the LKU and catalytic domains of known PI 4-kinases (Fig. 2A). The LKU domain in AtPI4Kβ was located close to the N terminus and was 20–37% identical to LKU domains from previously identified PI 4-kinases (Fig. 2B). The C terminus of AtPI4Kβ contained the putative catalytic domain, which was 43–57% identical to the corresponding domains from other PI 4-kinases (Fig. 2C). In addition, the A. thaliana protein described here, like all the members of subfamily 1.1, lacks a PH domain, a domain that characterizes members of subfamily 1.2. These observations indicated that AtPI4Kβ represented a new member of the subfamily 1.1 of PI 4-kinases, with a structure resembling most closely that of the yeast PIK1 protein. Overall, AtPI4Kβ was more similar to subfamily 1.1 PI 4-kinases than to the other A. thaliana partial PI 4-kinase, AtPI4Kα, and was most closely related to a putative PI 4-kinase from D. discoideum, with 30% identical and 52% similar amino acid residues.

Further sequence analyses showed that AtPI4Kβ also possessed a region similar to part of a sequence previously thought to be shared only by yeast PIK1 and a soluble 92-kDa PI 4-kinase from rat brain (Fig. 2D). The region common to these three proteins, designated NH, is approximately 90 amino acid residues in length.

Hydropathy analysis identified a relatively hydrophilic segment within the N-terminal half of AtPI4Kβ, whereas the rest of the protein had a more amphipathic character. Part of the hydrophilic segment, covering amino acids 239–560, contained a high percentage (46%) of charged amino acid residues, with 25% acidic (glutamate and aspartate) and 21% basic (lysine, arginine, and histidine) amino acids. This domain was not present in any of the previously identified PI 4-kinases. A more careful analysis of this region revealed the presence of a repeated motif. Fig. 3 shows that the repeated sequence, which occurs 11 times between amino acid position 212 and 508 (see Fig. 1A), has a length of 19–20 amino acids with a highly conserved inner core sequence of hydrophobic/positively charged/hydrophobic amino acids. This core sequence was N-terminal-flanked by acidic amino acids (aspartate or glutamate), and C-terminal-flanked by alternating positively and negatively charged amino acids.

**Biochemical Characterization of Recombinant AtPI4Kβ**—To investigate whether AtPI4Kβ encodes a functional PI 4-kinase, we expressed it with a polyhistidine tag at its N terminus in baculovirus-infected insect (S. frugiperda) cells. As shown in Fig. 4A, affinity-purified recombinant AtPI4Kβ appeared as a single band on a protein gel (Fig. 4A, left panel, lane 4). The purity of the recombinant protein was estimated to be higher than 95%, because almost no additional bands were present on the gel. Although it was not possible to detect recombinant AtPI4Kβ in the corresponding crude extract after Coomassie staining, an antibody directed against the N-terminal polypeptide tag showed that recombinant protein was present in the crude extract (Fig. 4A, right panel, lane 2). No cross-reacting protein was detected in cells expressing an unrelated protein without a polypeptide tag. The molecular mass of the recombinant AtPI4Kβ protein was estimated to be 125–130 kDa, which closely agrees with the calculated mass of 126 kDa.

When analyzed in vitro, recombinant AtPI4Kβ yielded a phospholipid that on TLC, co-chromatographed with Authentic PtdIns-4-P (Fig. 4B). The reaction product was deacylated and analyzed by HPLC on a Partisphere SAX column. The deacylated product co-eluted exactly with [3H]glycerophosphoinositol 4-phosphate after glycerophosphoinositol 3-phosphate (Fig. 4C). We also analyzed the levels of PtdIns-4-P in insect cells. Cells expressing the AtPI4Kβ consistently showed 25–30% in-
crease in PtdIns-4-P levels as compared with mock-infected cells (data not shown). These results confirm that AtPI4Kβ is a functional PI 4-kinase.

Triton X-100 activates animal PI 4-kinases (15) and is also able to activate the partially purified enzymes from carrot (44) and C. roseus suspension culture cells (45). The activity of recombinant AtPI4Kβ was almost unaffected by Triton X-100 concentrations below 0.3% (w/v) but increased 4- to 5-fold when the detergent concentration was increased to 0.6% (Fig. 5A).

Adenosine inhibits mammalian PI 4-kinases previously classified as type II more than it does type III enzymes (20). When tested on AtPI4Kβ, a moderate inhibitory effect was observed, i.e. 50% inhibition was reached at 200 μM adenosine (Fig. 5B).

Maximal activity of recombinant AtPI4Kβ protein was approximately 12 nmol/min/mg of protein (in the presence of 0.6% Triton X-100).

Fig. 1. Sequence analysis and gene structure of AtPI4Kβ. A, nucleotide and predicted amino acid sequences. Numbers on the right indicate amino acid positions. Note the presence of 11 repeated motifs (underlined) within the N-terminal half of the protein (see also text and Fig. 3). B, exon/intron structure of the AtPI4Kβ gene. Exons are presented as boxes. The four marked exons in the 3'9 region of the gene were identified via comparison with the AtPI4Kβ cDNA and were found to differ from the original computer prediction (see accession number AB008266). Numbers indicate exon sizes (nucleotides).
Wortmannin, a hydrophobic steroid-related compound from the fungus Talaromyces wortmannii, is a potent inhibitor of mammalian PI 3-kinases (46). Although PI 4-kinases were not first believed to be inhibited by wortmannin, it has recently been demonstrated that the yeast PI 4-kinase encoded by the STT4 gene is inhibited by approximately 95% at 10 nM wortmannin (47). Wortmannin-sensitive PI 4-kinases have also been cloned from human (48) and bovine (49), although sensitivity of the PI 4-kinase is at least 10 times lower than that of PI3-kinase. As is shown in Fig. 5C, wortmannin had little effect at concentrations up to 0.1 μM but inhibited the plant recombinant enzyme by approximately 90% at 10 μM.

Northern Blot Analyses Indicate Almost Constitutive Expression of the AtPI4Kβ Gene—The full-length AtPI4Kβ cDNA was used as hybridization probe to study transcript levels. A single transcript of approximately 4.5 kb was detected in leaves, roots, flowers, and stems of A. thaliana (Fig. 6, top left). Treatment of small plantlets with hormones, CaCl₂, or NaCl had no effect on AtPI4Kβ mRNA levels (Fig. 6, bottom). The AtPI4Kβ gene appeared therefore to be constitutively expressed in A. thaliana.

![Fig. 2. Structural organization of A. thaliana PI 4-kinase. A, protein domains present in AtPI4Kβ, in comparison with selected yeast and animal PI 4-kinases. Numbers indicate amino acid positions. Accession numbers are as follows: 92kPI4K (rat), D84667; ScPIK1 (yeast), F93104; PI4Ka (human), L36151; 230kPI4K (human), AF012872. B, multiple amino acid sequence alignment of the LKU domain of selected PI 4-kinases. Accession numbers: AtPI4Ka, AF035936; DdPI4K (D. discoideum), U23479; PI4Ka (human), U81802. C, multiple amino acid sequence alignment of the catalytic (Cat) domains. In B and C, amino acid residues identical in at least four sequences (conserved exchanges included) are indicated by gray and black shading. D, multiple amino acid sequence alignment of the novel homology (NH) domain present in A. thaliana AtPI4Kβ, yeast ScPIK1, and rat 92kPI4K.](image-url)
FIG. 3. Unique repetitive motif of AtPI4Kβ. Alignment of the 11 repeated motifs present between amino acid positions 212 and 508 (see also Fig. 1A). Red, acidic amino acids; blue, basic amino acids; green, hydrophobic amino acids.

DISCUSSION

Although PI 4-kinase activity has long been detected, characterized, and partially purified from higher plants, cloning and functional expression of a plant PI 4-kinase has not yet been reported. Here we describe the cloning of a cDNA from A. thaliana that encodes a functional 126-kDa PI 4-kinase. The size of this protein is similar to the size of the smaller of the PI 4-kinases isolated from yeast, D. discoideum, and animals, which is in the range of 90–122 kDa. Recently, Okpodu et al. (45) partially purified a soluble PI 4-kinase from carrot suspension culture cells. The molecular mass of this protein was estimated to be 83 kDa. Two distinct PI 4-kinase activities have recently been partially purified from spinach plasma membranes, with estimated sizes of 65 and 120 kDa.4 No peptide sequences for any of the partially purified plant PI 4-kinases are available. Therefore it is not possible to know whether any of the partially purified plant PI 4-kinases corresponds to homologs of AtPI4Kβ or AtPI4Kα.

Sequence analyses showed that AtPI4Kβ possesses the two conserved domains present in all PI 4-kinases, namely the LKU and the kinase catalytic domains, but lacked a PH domain. These structural features indicate that AtPI4Kβ is a new member of subfamily 1.1 of PI 4-kinases. The molecular structure of AtPI4Kβ most resembles that of the yeast PIK1 and the Dictyostelium DdPI4K proteins. We also identified a novel domain, NH, that corresponds to part of the sequence that was previously identified only in the yeast PIK1 protein and a rat soluble PI 4-kinase (22). Surprisingly, AtPI4Kβ is the only known PI 4-kinase to possess a unique repetitive motif constituted of 11 repeats of a charged core unit. Interestingly, three putative PtdIns-P kinases from Arabidopsis (Refs. 50 and 51 and accession number U95973) also contain a repeated motif, whereas none of the animal enzymes possess such a repeated motif. No clear function can be assigned to the LKU, the NH, or the domain with the repetitive motif; they may play a role in the interaction of PI 4-kinases with other proteins and/or membrane structures.

The partial PI 4-kinase isolated from A. thaliana, AtPI4Kα (30), possesses a PH domain, and its structure resembles that of members of subfamily 1.2 of PI 4-kinases. It was shown that the PH domain of AtPI4Kα can bind PtdIns-4-P and PtdIns-4,5-P2, but not PtdIns. It was suggested that AtPI4Kα, by binding phosphoinositides at its PH domain, could be involved in the regulation of actin polymerization. It appears, thus, likely that the PH domain of type 1.2 PI 4-kinases is not responsible for binding their substrate. Consequently, it is possible that substrate binding in PI 4-kinases is controlled by the LKU domain or a domain conserved structurally but not at the sequence level.

The catalytic properties of the recombinant plant protein, i.e. its stimulation by low concentrations of Triton X-100 as well as its moderate inhibition by adenosine, although they do not match exactly, still resemble those of the PI 4-kinases previ-

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4 T. Westergren, L. Ekblad, B. Jergil, and M. Sommarin, personal communication.
ously classified as type III more than those classified as type II. As recently observed with other PI 4-kinases, the AtPI4Kβ is inhibited by wortmannin at concentrations that are significantly higher than those required to inhibit PI 3-kinases. The residue corresponding to the Lys residue of the PI 3-kinase PI3Kα at which wortmannin binds covalently (52) is conserved in AtPI4Kβ as it is in all other cloned PI 4-kinases, including AtPI4Kα. Recently, wortmannin at high concentrations was shown to inhibit PI 3-kinase and PI 4-kinase activities in tobacco cells in vivo as well as protein sorting to the plant vacuole (53). These results suggest that the sorting of proteins to the plant vacuole is controlled by at least two different mechanisms, one of which is wortmannin-sensitive and may involve phosphoinositides (53). It has been shown in animal cells that a wortmannin-sensitive PI 4-kinase enzyme was involved in the formation of agonist-stimulated inositol trisphosphate (54). Because plants appear to express similar PI 4-kinase isoforms as animals, it will be of interest to test whether any of the factors that affect phosphoinositide levels in plants, such as light, mastoparan, and fungal elicitors (55), involve the response of a wortmannin-sensitive PI 4-kinase enzyme.

The sequences available so far indicate that in animals each of the two types of PI 4-kinases is represented by several splice variants of one single gene. The whole genome of *S. cerevisiae* contains only two PI 4-kinase genes. It is now clear that plants also contain subfamily 1.1 and subfamily 1.2 PI 4-kinases. Because PI 4-kinase activities have been detected in most cellular compartments, it is probable that the domains identified in PI 4-kinases are involved in the targeting of PI 4-kinase isoforms to various cellular compartments through interactions with other proteins or lipids. The similarity in structure of the PI 4-kinases found in the different phyla contrasts markedly with the current data available for PI-PLC. Three to four genes have been isolated from *A. thaliana* (56–58), potato (59), and soybean (60). Based on protein structure and biochemical properties, all plant PI-PLC isoforms appear to belong to a single family, which is most closely related to the d-type of mammalian PI-PLCs, but all lack a PH domain. Because phosphoinositides are now known to be important in processes not involving the canonical second messengers inositol trisphosphate and diacylglycerol, it will be of great interest to compare the regulation and function of PI 4-kinases between animal and plants. In addition, whereas the plant PI-PLC isoforms are expressed in tissue- and/or environmental-specific manner (56, 59), the plant PI 4-kinase reported here is ubiquitously expressed in the plant. Future experiments will tell whether plants possess additional subfamily 1.1 PI 4-kinase genes whose expression patterns are developmentally and/or environmentally regulated.

**Acknowledgments**—We thank Thomas Ehrhardt, Steffanie Hartje, and Sabine Zimmermann for help with the insect cell culture and Maryse Laloi for providing some of the RNA blots. We thank Gunnar Plesch, Nicola Weigmann, and Irina Staxen for critical comments on the manuscript. Charles Brearley thanks The British Council (British-German, ARC program) for support.

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