

# Patellin1, a Novel Sec14-Like Protein, Localizes to the Cell Plate and Binds Phosphoinositides<sup>1</sup>

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Membrane trafficking is central to construction of the cell plate during plant cytokinesis. Consequently, a detailed understanding of the process depends on the characterization of molecules that function in the formation, transport, targeting, and fusion of membrane vesicles to the developing plate, as well as those that participate in its consolidation and maturation into a fully functional partition. Here we report the initial biochemical and functional characterization of patellin1 (PATL1), a novel cell-plate-associated protein that is related in sequence to proteins involved in membrane trafficking in other eukaryotes. Analysis of the Arabidopsis genome indicated that PATL1 is one of a small family of Arabidopsis proteins, characterized by a variable N-terminal domain followed by two domains found in other membrane-trafficking proteins (Sec14 and Golgi dynamics domains). Results from immunolocalization and biochemical fractionation studies suggested that PATL1 is recruited from the cytoplasm to the expanding and maturing cell plate. In vesicle-binding assays, PATL1 bound to specific phosphoinositides, important regulators of membrane trafficking, with a preference for phosphatidylinositol(5)P, phosphatidylinositol(4,5)P<sub>2</sub>, and phosphatidylinositol(3)P. Taken together, these findings suggest a role for PATL1 in membrane-trafficking events associated with cell-plate expansion or maturation and point to the involvement of phosphoinositides in cell-plate biogenesis.

Plant cells partition their cytoplasm during cytokinesis by building a new cell wall from the inside-out between the two sets of daughter chromosomes (Staelin and Hepler, 1996; Heese et al., 1998; Smith, 1999; Otegui and Staelin, 2000a; Assaad, 2001). Construction of this new partition is dominated by membrane-trafficking events (Bednarek and Falbel, 2002). The process begins when the phragmoplast, a plant-specific cytoskeletal array, is assembled from the remnants of the mitotic spindle in late anaphase and guides Golgi-derived vesicles carrying cell wall materials to the plane of division. There they fuse to initiate formation of the cell plate, a transient disc-shaped membranous network that undergoes a complex series of transformations over the course of its development (Samuels et al., 1995; Segui-Simarro et al., 2004). The cell plate expands centrifugally by the addition of vesicles to its periphery, accompanied by depolymer-

ization of phragmoplast microtubules from the center and their repolymerization at the edge (Zhang et al., 1990; Asada et al., 1991). An actomyosin-dependent mechanism guides the expanding cell plate to a previously determined cortical division site where it fuses with the parent cell membrane (Valster et al., 1997; Granger and Cyr, 2001; Molchan et al., 2002). Upon fusion, fenestrae in the plate are filled in by the addition of new vesicles. A complex maturation process, involving membrane recycling and cellulose biosynthesis, yields a fully functional and integrated partition (Samuels et al., 1995; Moore and Staelin, 1998; Nickle and Meinke, 1998; Zuo et al., 2000; Segui-Simarro et al., 2004).

While the events of plant cytokinesis are known in detail at an ultrastructural level (Samuels et al., 1995; Otegui and Staelin, 2000b, 2004; Otegui et al., 2001; Segui-Simarro et al., 2004), our understanding at the molecular level is quite limited. Significant progress has been made, however, by analysis of mutants that are defective in construction of the cell plate, typified by multinucleate cells and cell wall stubs and by studies of proteins that localize to the cell plate. Not surprisingly, many of the molecules that function in cell-plate biogenesis are related to proteins involved in membrane-trafficking in other eukaryotes (Bednarek and Falbel, 2002). Several kinesin-related proteins function specifically during cytokinesis. AtPAKRP2, which localizes to brefeldin A-sensitive puncta during early plate development, is a likely candidate for the motor that drives vesicle movement along the phragmoplast microtubules (Lee et al., 2001; Smith, 2002),

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and the kinesin-related protein HINKEL/NACK1 functions in microtubule dynamics during plate expansion in collaboration with a mitogen-activated protein kinase-signaling pathway (Nishihama et al., 2002; Strompen et al., 2002). Proteins implicated in vesicle formation and membrane remodeling during cytokinesis include dynamin-like proteins such as phragmoplastin in soybean (*Glycine max*; Gu and Verma, 1996, 1997) and ADL1A and ADL1E, which play an essential role in cytokinesis and polar cell growth in *Arabidopsis* (Kang et al., 2001, 2003). A growing number of SNAREs and other proteins involved in vesicle docking/fusion events localize to the cell plate and are also critical for normal cytokinesis. These include the SNAREs, KNOLLE (Lukowitz et al., 1996; Lauber et al., 1997), and AtSNAP33 (Heese et al., 2001) and KEULE, a Sec1 family protein likely to function in SNARE complex regulation (Assaad et al., 2001). In addition, the novel plant SNARE, NSPN11, localizes primarily to the developing cell plate and cofractionates with KNOLLE, evidence that it too is involved in vesicle fusion events at the cell plate (Zheng et al., 2002). AAA proteins (ATPases associated with various cellular activities) that regulate vesicle fusion events by disassembling the SNARE complexes to allow for subsequent rounds of fusion (May et al., 2001) also appear to be important for membrane traffic at the cell plate. The *Arabidopsis* ortholog of one of these, CDC48, colocalizes at the cell plate with KNOLLE and another plate-associated SNARE, SYP31. KNOLLE was also detected in a complex with a Sec18p/NSF AAA protein (Rancour et al., 2002). GTP-binding proteins of two families that function in membrane trafficking, Rho and Rab, have also been implicated in cell-plate biogenesis. Rho-related GTPases localize to the cell plate (Molendijk et al., 2001), and the *scd1* mutant of *Arabidopsis*, which displays defects in guard cell cytokinesis, accompanied by the accumulation of secretory vesicles, contains a domain found in other proteins that interact with Rab proteins (Falbel et al., 2003).

We report here the identification and initial characterization of patellin1 (PATL1), a novel cell-plate-associated protein that is related in sequence to proteins involved in membrane trafficking in other eukaryotes. Based on its cell-plate localization we have chosen the name patellin from the Latin "patella," which means "small plate." PATL1 is one of a small family of *Arabidopsis* proteins characterized by two domains found in other membrane-trafficking proteins, a Sec14 lipid-binding domain and a Golgi dynamics (GOLD) domain. GOLD domains are found in a diverse group of proteins involved in Golgi function and vesicle traffic, where they are thought to participate in protein-protein interactions (Anantharaman and Aravind, 2002). Sec14p is the defining member of one of two families of eukaryotic proteins, originally defined by their ability to transfer phosphatidylinositol (PtdIns) and/or phosphatidylcholine (PtdCh) monomers between membrane bilayers (Allen-Baume et al.,

2002). Sec14p, first identified in yeast (*Saccharomyces cerevisiae*), plays an essential role in the formation and exit of secretory vesicles from the trans-Golgi network (TGN; Bankaitis et al., 1989, 1990). The second family of these so-called PtdIns lipid transfer proteins (PITPs) is specific to animal cells and includes PITP $\alpha$ , the defective protein in mice with the vibrator neurodegenerative disorder (Hamilton et al., 1997), and the retinal degeneration protein B of *Drosophila* (Milligan et al., 1997). PITPs and related proteins of both families play diverse roles in membrane trafficking, the regulation of phospholipid metabolism, and phosphoinositide-signaling pathways (Kearns et al., 1998a; Li et al., 2000b; Hsuan and Cockcroft, 2001; Allen-Baume et al., 2002).

Proteins related to Sec14p have more recently been discovered in plants, mammals, and fungi. Yeast contains four partially redundant SEC14 homologs (SFH2, SFH3, SFH4, and SFH5) that play additional roles including the activation of phospholipase D and perhaps phosphoinositide and sterol production (van den Hazel et al., 1999; Li et al., 2000a; Wu et al., 2000). In mammalian cells the Sec14 family includes proteins that bind and/or transfer  $\alpha$ -tocopherol- and cis-retinaldehyde and a supernatant protein factor that regulates cholesterol synthesis (Stocker and Baumann, 2003). In addition, mammalian cells contain a growing list of proteins that contain a Sec14 lipid-binding domain, including the human MEG2 protein tyrosine phosphatase that induces vesicle fusion in T cells and exchange factors and activating proteins for Rho family GTPases including members of the multifunctional Dbl family (Gu et al., 1992; Aravind et al., 1999; Huynh et al., 2003). In plants, several SEC14-like proteins have been described, and they all exhibit approximately 25% amino acid identity with Sec14p and complement the *sec14-1<sup>ts</sup>* mutant. The soybean proteins, Ssh1p and Ssh2p (soybean SEC14 homolog-1 and -2), are distinct from Sec14p in their lipid transfer and binding activities (Kearns et al., 1998b). Ssh1p functions during a hyperosmotic stress-induced signaling pathway and regulates the activity of PtdIns kinases, while the function of Ssh2p is yet to be determined (Monks et al., 2001). The *Arabidopsis* *sec14*-like protein, AtSEC14, which was cloned by screening for sequences that rescued the yeast *sec14-1<sup>ts</sup>* mutant, also transfers PtdIns (Jouannic et al., 1998). Although its biological function is unknown, recent studies are consistent with a role in vesicle trafficking; AtSEC14 was shown to copurify on a naphthylphthalamic acid affinity resin with proteins involved in vesicle traffic between the TGN, plasma membrane, and endocytic compartments, including ADL1 and  $\beta$ -adaptin (Murphy et al., 2002). Nitrogen-fixing root nodules also contain Sec14-like PITPs. A nodule-specific PITP gene family was identified in Lotus that is subject to elaborate transcriptional control during nodule development, where these proteins may function in lipid-signaling pathways that regulate membrane biogenesis during nodulation (Kapranov et al., 2001).

In this study we provide evidence for the involvement of a novel Sec14-like protein, PATL1, in plant cytokinesis. We show that PATL1 localizes to the somatic cell plate coincident with its expansion and maturation. We also show that PATL1 is a peripheral membrane protein that binds phosphoinositides with a preference for PtdIns(5)P, PtdIns(4,5)P<sub>2</sub>, and PtdIns3P. Taken together these results suggest a role for PATL1 in membrane-trafficking events associated with cell-plate expansion and maturation and point to the involvement of phosphoinositides in cell-plate biogenesis.

## RESULTS

### The Arabidopsis PATL Gene Family

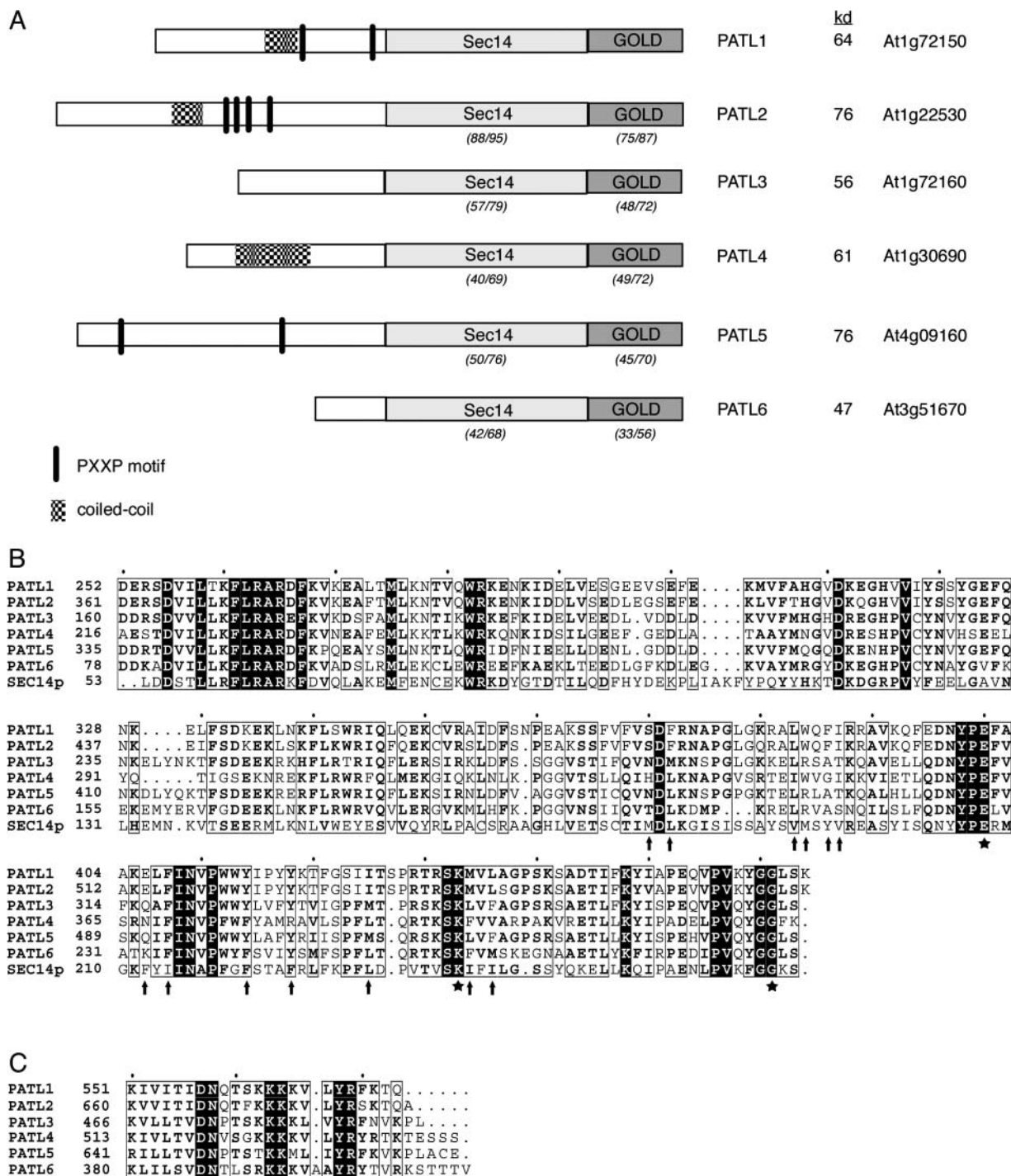
PATL1 was originally identified from a tryptic peptide sequence of a protein that copurified with a zucchini (*Cucurbita pepo*) membrane-associated F-actin-binding protein. Using this peptide sequence we cloned and sequenced the corresponding full-length PATL1 cDNA from Arabidopsis using standard reverse transcription-PCR methods. A search of the Arabidopsis genome database at the National Center for Biotechnology Information (NCBI) identified five additional proteins that show significant similarity in their overall amino acid sequence (27%–57% identity/44%–67% similarity) and protein domain structure (Fig. 1). Expressed sequence tag (EST)/cDNAs have been reported in the public databases for all of the PATLs, indicating that all members of the gene family are expressed. Furthermore, transcription of PATL1, PATL2, PATL3, PATL4, and PATL6 was confirmed by whole genome array analysis (Yamada et al., 2003). The predicted PATL proteins (PATL1, At1g72150; PATL2, At1g22530; PATL3, At1g72160; PATL4, At1g30690; PATL5, At4g09160; and PATL6, At3g51670) range in mass from 47 to 76 kD.

The domain structure of the Arabidopsis PATLs is shown in Figure 1A. The PATL proteins possess an N-terminal region that is variable in length (81–357 amino acids) and sequence composition. In all of the family members, except for PATL6, this domain is very acidic (pI approximately 4) with an unusually high percentage of charged amino acids (30%–48%), especially Glu (19%–27%). The N termini of PATL1, PATL2, and PATL4 contain numerous EEK repeats, reminiscent of those found in the neurofilament triplet H proteins (Herrmann and Aebi, 2000). Their N-terminal sequences also predict a coiled coil, a common protein oligomerization-folding motif (Burkhard et al., 2001). In addition, the N termini of PATL1 and PATL5 contain several PXXP sequences consistent with binding to SH3 protein interaction domains (Feng et al., 1994). SH3 domains participate in protein-protein interactions involved in cytoskeletal dynamics, tyrosine kinase-signaling pathways, and vesicular trafficking (Kay et al., 2000).

Analysis with InterProScan (<http://www.ebi.ac.uk/InterProScan/>) revealed that the PATLs belong to the Sec14 family of PITPs. The variable N-terminal domain of the PATLs is followed by a region of similarity to the yeast PITP, Sec14p (approximately 26%/48% amino acid similarity/identity; Fig. 1B). The SEC14 domain of the PATLs includes a region of similarity to the last 74 residues of the N terminus of Sec14p followed by its C-terminal phospholipid-binding pocket. The small  $\alpha$ -helical N-terminal domain of Sec14p contains most of the first 129 residues known to be sufficient for its stable association with Golgi membranes (Sha et al., 1998). The PATLs, however, are missing the first 55 residues of this region. The SEC14 domains of PATL1 and PATL2 are the most closely related to each other, with 88% amino acid sequence identity and 95% similarity. In contrast, the SEC14 domains of the other PATLs show only 40% to 57% identity and 69% to 79% similarity when compared to PATL1.

An alignment of the PATL SEC14 domain sequences with the C-terminal lipid-binding pocket of Sec14p suggested that the PATLs may interact with phospholipids (Fig. 1B). The Sec14p residues that have been shown by mutational analysis to be critical for PtdIns-binding and/or transfer (E207, K239, and G266) are perfectly conserved in all of the PATLs. The molecular basis of the sec14-1<sup>ts</sup> allele is a G266D substitution, which results in destabilization of the hydrophobic pocket (Cleves et al., 1989), while E207 and K239 form a salt bridge that has been conserved in all Sec14 proteins that either bind or transfer PtdIns (Li et al., 2000b). Moreover, many of the hydrophobic amino acids that line the hydrophobic pocket of Sec14p (M177, L179, V190, M191, Y193, V194, F212, I214, F221, F225, L232, I240, and L242) are also conserved in the PATLs (Sha et al., 1998; Sha and Luo, 1999). This is particularly striking for PATL1 and PATL2, where all but two of these sites are occupied by hydrophobic residues.

Analysis of the PATL sequences with the ScanProsite program (<http://ca.expasy.org/tools/scanprosite>) revealed that all members of the family also contain a conserved C-terminal GOLD domain. GOLD domains are found in a number of eukaryotic proteins involved in Golgi function and lipid traffic including the p24 family of proteins involved in traffic between the endoplasmic reticulum and Golgi, the yeast cytoplasmic oxysterol-binding protein, Osh3p, the human Golgi protein, GCP60, and animal Sec14-like proteins (Anantharaman and Aravind, 2002). The very C-terminal end of the GOLD domain of the PATLs is Lys rich and contains a conserved motif, K(X<sub>10</sub>)(K/R) KKK/M( $\Phi_{2-3}$ ) YR, that is reminiscent of the PtdIns(4,5)P<sub>2</sub>-binding motif, K(X<sub>9</sub>) KX(K/R)(H/Y), found in two proteins involved in clathrin-coated vesicle formation, AP180 and  $\mu$ 2-adaptin (Fig. 1C; Ford et al., 2001; Mao et al., 2001; Rohde et al., 2002). As is true for their Sec14 domains, the GOLD domains of PATL1 and PATL2 are most closely related with 75% identity and 87% simi-



**Figure 1.** Arabidopsis PATL protein structures. A, Diagram of the domain structure of Arabidopsis PATLs. The percentage amino acid identity/similarity between the Sec14 and GOLD domains of PATL1 and the other patellin family members is shown in parentheses below the domains. Pairwise analysis was performed using ClustalW with a gap opening penalty of 10 and a gap extension penalty of 0.2. The molecular masses predicted from the open reading frame sequences are shown. B, Amino acid sequence alignments of the Sec14 domain of the Arabidopsis PATLs and yeast Sec14p. Sec14p residues involved in PtdIns transfer activity are marked with a star, while hydrophobic residues that line the lipid-binding pocket are indicated by arrows. C, Amino acid sequence alignments of the C-terminal Lys-rich motif of the PATLs.

ilarity in this region. In contrast, the GOLD domains of the other PATLs show only 33% to 49% identity and 56% to 72% similarity when compared to PATL1.

### Production and Characterization of PATL1 Protein and Antibodies

Recombinant PATL1 was produced in *Escherichia coli* for use in lipid-binding assays and the production of polyclonal antisera. PATL1 was expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein in the vector pGEX6P-1 (Amersham Biosciences, Piscataway, NJ; Fig. 2A, lanes 1 and 2). The resulting fusion protein was affinity purified on GST-Sepharose (Amersham Biosciences; Fig. 2A, lane 3), cleaved with PreScission protease (Amersham Biosciences), and affinity purified again to remove the GST tag (Fig. 2A, lane 4). Surprisingly, the purified PATL1 has an apparent

molecular mass of 105 kD on SDS-PAGE, while its predicted mass is only 64 kD. This discrepancy is most likely due to poor binding of SDS by the acidic N-terminal domain. Anomalous migration of acidic proteins in SDS gels is common (Armstrong and Roman, 1993). Furthermore, a truncated PATL1 that lacks the acidic N-terminal domain does not show this anomalous migration (data not shown). A protein that consists only of the C-terminal 190 amino acids of PATL1 was also expressed and purified (data not shown).

Both the full-length and GOLD domain proteins were used for antibody production in rabbits. The resulting antibodies to the full-length protein reacted with a single band of 105 kD in immunoblots of Arabidopsis root extracts (Fig. 1B, lane 1), which comigrated with the bacterially expressed PATL1 (Fig. 1A, lane 4). The antiserum raised against the GOLD domain also reacted with a 105-kD protein; however, significant cross reaction to several smaller proteins was also seen (Fig. 1B, lane 2).

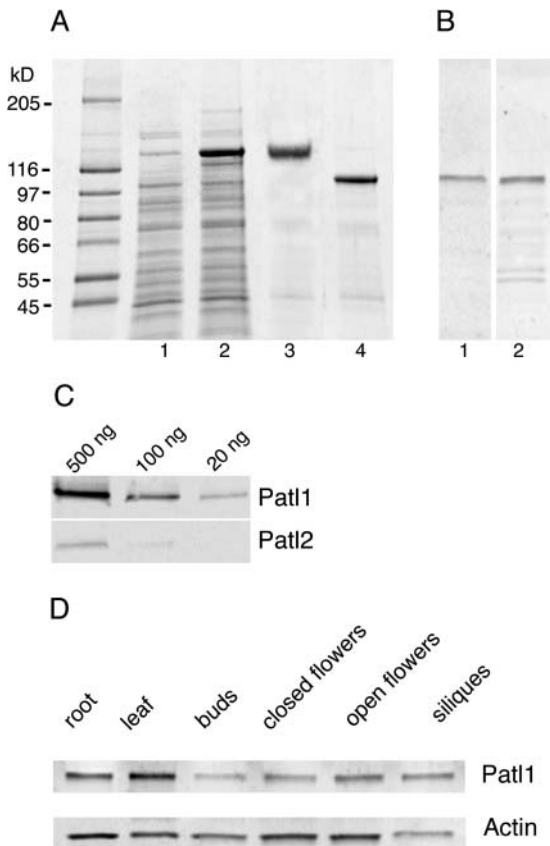
Occasionally, an additional very faint band with an apparent molecular mass of 125 kD was detected in tissue extracts with the antiserum to full-length PATL1, most likely due to a weak cross reaction with PATL2 (data not shown). To test directly for cross reaction with PATL2 (the PATL family member most similar in sequence to PATL1), immunoblots of PATL1- and PATL2-GST fusion proteins were probed with anti-PATL1. The results, shown in Figure 2C, revealed a very weak cross reaction; the signal obtained with only 20 ng of PATL1 was similar to that seen with 500 ng of PATL2, indicating an approximately 25-fold difference in sensitivity. We believe it is highly unlikely that the PATL1 antiserum cross-reacts with PATL3, 4, 5, and 6, given the high degree of sequence divergence between these antigens. Given its high degree of specificity, the antiserum to the full-length PATL1 was used for the remaining experiments.

### PATL1 Is Ubiquitously Expressed in Arabidopsis Organs

Immunoblots of protein extracts of Arabidopsis organs were performed to determine the distribution of the PATL1 throughout the plant (Fig. 2D). An actin antibody was used as a positive control. PATL1 was detected in all tissues examined, including young roots and leaves, flowers at different stages of development, and green siliques. PATL1 was most abundant in expanding leaves and roots. PATL1 levels were low in flower buds but increased severalfold during flower development. The protein was also detected in developing siliques.

### PATL1 Is a Peripheral Membrane Protein

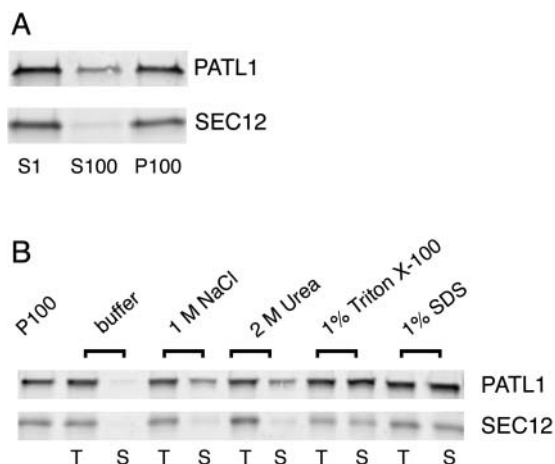
Biochemical fractionation studies were performed to determine the intracellular distribution of PATL1. A postnuclear supernatant (S1) prepared from Arabidopsis roots was ultracentrifuged at 100,000g to pro-



**Figure 2.** PATL1 antibody production and characterization. The Arabidopsis PATL1 coding region was expressed as a GST-fusion in *E. coli* and purified for biochemical analysis and antibody production. A, SDS-PAGE of uninduced (lane 1) and induced (lane 2) *E. coli* extracts, purified GST-patellin (lane 3), and cleaved fusion protein (lane 4). Molecular mass markers are shown at left. B, Immunoblots of Arabidopsis root total protein extracts (10  $\mu$ g/lane) probed with PATL1 antisera raised against full-length protein (lane 1) or the GOLD domain (lane 2). C, Immunoblots of PATL1 and PATL2-GST fusion proteins probed with PATL1 antibody. D, Immunoblots of Arabidopsis organ extracts (10  $\mu$ g/lane) probed with PATL1 or actin antibodies.

duce soluble cytosolic (S100) and microsomal membrane (P100) fractions. The resulting fractions were then analyzed by immunoblots using the antibodies to the full-length PATL1 as well as an integral membrane marker, AtSEC12 (Bar-Peled and Raikhel, 1997; Fig. 3A). While PATL1 was found in both the soluble and membrane fractions, the majority of the protein (approximately 80%) was associated with the membrane. AtSEC12, an integral membrane protein marker, was found in the P100 fraction as predicted. These results suggested that PATL1 exists in a cytoplasmic pool that can associate peripherally with intracellular membranes.

To determine the biochemical nature of the interaction of PATL1 with the membrane, we extracted samples of the pelleted membranes (P100) with buffers containing 1 M NaCl, 2 M urea, 1% (v/v) Triton X-100, or 1% (w/v) SDS (Fig. 3B). Both PATL1 and the integral membrane protein, AtSEC12, were completely solubilized by the detergent treatments. Approximately 50% of the PATL1 was also extracted by 1 M NaCl or 2 M urea, consistent with a peripheral membrane association. Also consistent with this conclusion is the observation that PATL1 was almost completely extracted by 0.1 M NaOH (data not shown). Partial extractability by either NaCl or urea suggests that both electrostatic interactions and hydrogen bonds stabilize the PATL1 interaction with the membrane.



**Figure 3.** PATL1 is a peripheral membrane protein. A, Immunoblots of Arabidopsis root subcellular fractions. Arabidopsis root extracts were centrifuged at 1,000g to yield a total cell extract (S1). The S1 fraction was centrifuged at 100,000g to yield soluble (S100) and membrane (P100) fractions. The S1, S100, and P100 fractions were analyzed by immunoblotting with antibodies to PATL1 and the integral membrane marker SEC12. Similar results were obtained in three independent experiments. B, Samples of the P100 fraction were washed in the indicated solutions. A total sample (T) was collected prior to centrifugation at 100,000g to yield the solubilized protein (S). Membrane equivalents of the T and S samples were analyzed by immunoblotting with antibodies to PATL1 or SEC12. Similar results were obtained in two independent experiments.

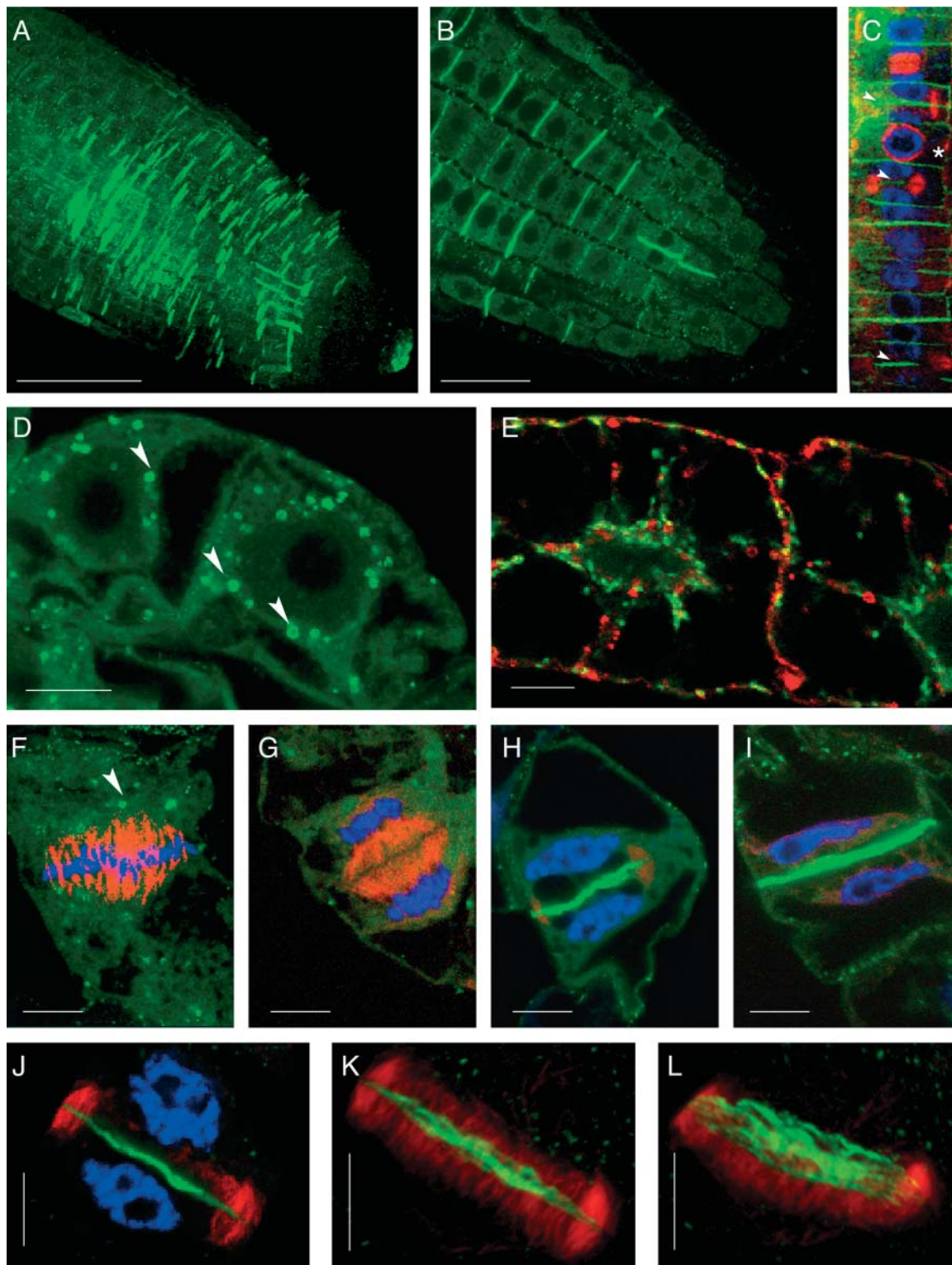
### PATL1 Localizes to the Cell Plate in Arabidopsis Roots

Immunolocalization studies of Arabidopsis roots were performed to provide insight into the cellular function of PATL1. Labeling was not detected with the preimmune serum (data not shown). Diffuse cytoplasmic staining for PATL1 was detected throughout the root tip, with the exception of the meristematic zone (Fig. 4A). Within the elongation zone, where newly born cells continue to divide and begin expansion, labeling was most intense on recently completed cell plates and punctate cytoplasmic structures (Fig. 4, A and B). PATL1 label was strongest on approximately every other transverse wall, consistent with persistence of PATL1 at the recently completed cell plates for a time following the completion of cytokinesis. Consequently, the PATL1 labeling pattern appears to provide a record of recently completed cell divisions.

To facilitate identification of PATL1's time of arrival at the cell plate, roots were triple labeled for PATL1, tubulin, and nuclei. In the file of rapidly dividing cortical cells shown in Figure 4C, PATL1 label was most pronounced at cell plates in which the microtubules had been displaced to the periphery of the centrifugally expanding phragmoplasts (marked with arrows). These results suggest a role for PATL1 in the later stages of cell-plate formation.

### PATL1 Localization in Dividing Tobacco BY2 Cells

Immunolocalization studies of tobacco (*Nicotiana tabacum*) BY2 cells revealed that they too contain a cell-plate-associated PATL1 antigen. Logarithmically growing BY2 cells were chemically fixed and immunostained for PATL1 and tubulin and stained for DNA with TO-PRO-3. In interphase (Fig. 4D) and metaphase (Fig. 4F) cells, PATL1 was detected in the cytoplasm and in punctate Golgi-sized structures, many of which showed a doughnut-like pattern similar to that previously described for Golgi labeled with a Golgi-targeted green fluorescent protein (GFP; GmMan1::GFP; Nebenfuhr et al., 1999). In those experiments the "doughnuts" were thought to represent a face on view of Golgi stacks where the GmMan1::GFP fusion protein was restricted to the rim of the cisternae. During metaphase the PATL1-labeled structures accumulated at the spindle poles (Fig. 4F) as has been reported for Golgi (Nebenfuhr et al., 2000). To determine if the PATL1-labeled structures are in fact Golgi, we performed immunolocalization experiments using BY2 cells with a Golgi-targeted GFP (GmMan1::GFP; Nebenfuhr et al., 1999). The results, shown in Figure 4E, revealed that PATL1 does not localize to the Golgi; there was no colocalization of PATL1 with GmMan1::GFP. The PATL1-labeled structures may represent endosomal compartments. Ring-like structures have been reported for the early endosomal compartments labeled by the Rab GTPases, Ara6, and Ara7 (Ueda et al., 2001).



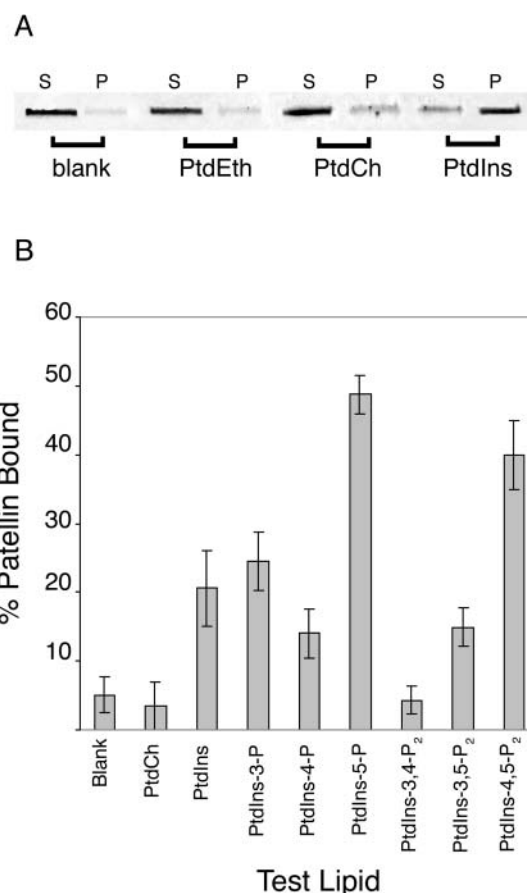
**Figure 4.** Immunolocalization of PATL1 in Arabidopsis roots and tobacco BY2 cells. Arabidopsis roots were immunolabeled and imaged by confocal microscopy. A, Confocal projection compiled from 1  $\mu\text{m}$  sections through an Arabidopsis root tip shows cell-plate labeling for PATL1 (green) in the zone of cell division. B, Single optical section from the projection shown in A shows PATL1 staining of cell plates and punctate cytoplasmic structures. Bars in A and B = 20  $\mu\text{m}$ . C, Colocalization of PATL1 (green) with tubulin (red) and DNA (blue) in a file of cortical cells. Arrowheads mark the sites of PATL1 staining of expanding cell plates. The asterisk marks a cell with a preprophase band. D, Interphase cells stained for PATL1 (green). E, Interphase cells expressing Golgi-associated GmMan1::GFP (green) and immunostained for PATL1 (red). F to L, Colocalization of PATL1 (green) with tubulin

An examination of the cell plate at different stages of development (Fig. 4, G–I) revealed that PATL1 label first appeared at the plate during late telophase. PATL1 label was not detected at the cell plate of early telophase cells in which remnants of the spindle microtubules were still evident (Fig. 4G) but was observed in late telophase cells in which the phragmoplast was undergoing radial expansion with concomitant displacement of the microtubules to the periphery (Fig. 4, H and J). Many of the expanding phragmoplasts we observed, like the one shown in Figure 4H, were anchored at one side of the cell as has been previously described (Culter and Ehrhardt, 2002). Staining of the newly completed cell plate persisted for a time posttelophase and was typically observed in cells in which the two new daughter nuclei were still close to the recently completed partition (Fig. 4I). These results are similar to those observed in *Arabidopsis* roots and are consistent with a model in which PATL1 is recruited from the cytoplasm to the division plane at the time of cell-plate expansion.

To determine if the plate-associated PATL1 was evenly distributed throughout the plate or concentrated at the site of new vesicle addition at the periphery, we performed a three-dimensional computer reconstruction of a cell plate undergoing phragmoplast expansion (Fig. 4J). Projections of this reconstruction, shown in Figure 4, K and L, revealed that PATL1 is distributed across the plate and is fibrillar in nature. The signal was weakest at the periphery. Thus, PATL1 is not concentrated where new vesicles are being added to the expanding plate but rather in the region of the plate where the maturation process is about to begin (Samuels et al., 1995; Segui-Simarro et al., 2004). These results are in marked contrast to those observed for proteins, such as AtPAKRP2 (Lee et al., 2001) and KNOLLE (Lauber et al., 1997) involved in the initial vesicle traffic to the plate and early vesicle fusion events, respectively.

### PATL1 Binds Phosphoinositides

We were interested to explore the ability of PATL1 to interact with phospholipids, given the presence of the SEC14 phospholipid-binding domain and Lys-rich C-terminal motif. We first tested the ability of PATL1 to bind to unilamellar membrane vesicles composed of phosphatidylethanolamine (PtdEth), PtdCh, or PtdIns alone. Vesicle-bound PATL1 was separated from unbound protein by centrifugation of the reaction mixture at 164,000g. PATL1 in the supernatant (unbound)



**Figure 5.** PATL1 binds phosphoinositides. Vesicle cosedimentation assays were used to test PATL1 protein for phospholipid-binding activity. A, PATL1 (2.5  $\mu$ g) was mixed with unilamellar vesicles composed of 25 mM PtdEth, PtdCh, or PtdIns. Vesicle bound proteins (P) were separated from unbound proteins (S) by centrifugation at 164,000g and analyzed by fluorescence scanning of SYPRO red-stained SDS gels. PATL1 protein alone was used as a blank. B, PATL1 binding to phosphoinositides was tested as described above except that unilamellar vesicles were made from a mixture of 10:1 PtdEth:test phosphoinositide and the lipid concentration was 2.2 mM. The percentage of patellin bound was quantified using ImageQuant software. Values are the mean  $\pm$  SE from at least three different experiments.

and the pellet (vesicle-bound) was then analyzed on SDS gels (Fig. 5A). To control for nonspecific pelleting of the protein, a blank with protein alone was run. PATL1 did not bind to the PtdEth vesicles but did bind to vesicles of PtdCh and PtdIns, both phospholipids transferred by yeast Sec14p. Under the conditions reported here, approximately 10% of the PATL1 bound to the PtdCh vesicles while over 90% bound to vesicles made of PtdIns.

### Figure 4. (Continued.)

(red) and DNA (blue). Single optical sections midway through the division plane are shown in F to J. Interphase (D) and metaphase (F) cells show PATL1 labeling of 1  $\mu$ m cytoplasmic structures (marked with arrowheads). Early- (G), late- (H), and post- (I) telophase cells show PATL1 staining of the developing cell plate. Three-dimensional computer reconstructions built from 0.2- $\mu$ m sections through the top half of the phragmoplast shown in J are projected at 10° and 20° from the optical axis in K and L, respectively. These images show that PATL1 at the plate is fibrillar in structure. Bars in D to L = 10  $\mu$ m.



PATL1's preference for PtdIns raised the possibility that it might bind to derivatives of PtdIns as well. The D-3, D-4, and D-5 hydroxyl groups of the inositol head group of PtdIns can be phosphorylated separately or in combination at each of these positions to produce seven possible derivatives (Fruman et al., 1998; Anderson et al., 1999). All of these, with the exception of PtdIns(3,4,5)P<sub>3</sub>, have been found in plant cells (Munnik et al., 1998; Stevenson et al., 2000; Mueller-Roeber and Pical, 2002). These so-called phosphoinositides have emerged as important regulators in a number of cellular activities including vesicle-trafficking and cytoskeletal dynamics, both processes that are central to cell-plate biogenesis. Consequently, we were very interested to test PATL1 for phosphoinositide-binding activity. For these experiments unilamellar vesicles were made from PtdEth spiked with the test phosphoinositide in a 10:1 molar ratio. A 10-fold lower amount of total lipid was employed. The relative amounts of PATL1 in pellets and supernatants were quantified and expressed as a percentage of the total PATL1 in the assay (Fig. 5B). Substantial binding (49%) was detected to PtdIns(5)P, a phosphoinositide just recently shown to exist in plants (Meijer et al., 2001). The function of PtdIns(5)P is relatively unexplored at this time although there is evidence that it is involved in osmotic stress responses in both mammalian cells and *Chlamydomonas* (Meijer et al., 2001; Sbrissa et al., 2002). In mammalian cells, PtdIns(5)P can also serve as a precursor to PtdIns(4,5)P<sub>2</sub>, a possibility that is yet to be explored in plant cells (Rameh et al., 1997).

A similar level of PATL1 binding (40%) was also detected to vesicles containing PtdIns(4,5)P<sub>2</sub>. PtdIns(4,5)P<sub>2</sub> is a substrate for phospholipase C, which produces Ins(3,4,5)P<sub>3</sub> and diacylglycerol, signals that release calcium intracellular stores and activate protein kinase C, respectively (Berridge, 1993). PtdIns(4,5)P<sub>2</sub> is also an important regulator in its own right, directly regulating proteins involved in a diversity of cellular processes. Of particular interest here are its functions in actin cytoskeletal dynamics and membrane trafficking (Toker, 1998). An intermediate level of binding was observed for vesicles that contained PtdIns (21%) and PtdIns(3)P (25%). PtdIns(3)P functions in vesicle traffic from the TGN to the vacuole in both yeast and plants and has been implicated in traffic from the TGN to the lysosome in mammalian cells (Matsuoka et al., 1995; Corvera et al., 1999; Kim et al., 2001). Low, but significantly above background, binding was observed with PtdIns(4)P and PtdIns(3,5)P<sub>2</sub>. PATL1 did not bind to vesicles that contained PtdIns(3,4)P<sub>2</sub>.

## DISCUSSION

We have reported here the identification and initial biochemical characterization of PATL1, one of a small family of novel *Arabidopsis* proteins characterized by

the presence of a Sec14 and a GOLD domain, both domains found in membrane-trafficking proteins in other eukaryotes. We have shown that PATL1 localizes to the cell plate at the time of its expansion and maturation, a site of membrane traffic. In addition, we have demonstrated that PATL1 binds to specific phosphoinositides, important regulators of membrane-trafficking and cytoskeletal dynamics. Based on its conserved domain structure, localization patterns, and biochemical properties, we hypothesize that PATL1 plays a role in membrane-trafficking events during the later stages of cytokinesis.

The presence of the Sec14 and GOLD domains in PATL1, especially the striking conservation of the Sec14 lipid-binding pocket, strongly suggests a role in vesicle trafficking. In yeast, Sec14p catalyzes the transfer of PtdIns, and to a lesser extent PtdCho, between membrane compartments *in vitro* although the exact relationship between this activity and its *in vivo* role in protein secretion is unclear (Phillips et al., 1999). A wealth of *in vivo* studies, however, are consistent with a complex function for Sec14p in coordinating Golgi phospholipid metabolism with membrane traffic from the Golgi (Li et al., 2000b). Sec14p influences the lipid composition of the Golgi membrane in several ways including the following: (1) acting as a sensor of the PtdIns/PtdCho ratio in Golgi membranes (Cleves et al., 1991; Skinner et al., 1995); (2) regulating the levels of PtdIns(4)P (Whitters et al., 1993; Hama et al., 1999; Phillips et al., 1999; Stock et al., 1999); and (3) influencing phospholipase D activity, which in turn effects the phosphatidic acid and diacylglycerol levels in the membrane (Sreenivas et al., 1998; Xie et al., 1998). Recent studies suggest that Sec14p produces a Golgi membrane lipid environment required for activation of the Arf GTPase-activating proteins, Gcs1p, and Age2p, during vesicle biogenesis (Yanagisawa et al., 2002). The presence of the Sec14p-lipid-binding domain in PATL1 raises the intriguing possibility that PATL1, like Sec14p, may promote vesicle formation by influencing donor membrane lipid composition. PATL1's additional domains and distinctive phosphoinositide-binding specificities, however, also make novel functions likely.

The GOLD domain in the PATL1 C terminus also suggests a role in vesicle trafficking. GOLD domains, which are found in a number of proteins involved in Golgi dynamics and membrane traffic, are thought to mediate protein-protein interactions with a variety of target proteins. Proteins with GOLD domains have been classified into two groups. The first group, the p24 proteins, regulates cargo selection and packaging into the coated vesicles that traffic between the endoplasmic reticulum and Golgi (Anantharaman and Aravind, 2002). These proteins contain a central trans-membrane domain with a C-terminal region that interacts with vesicle coat proteins and an N-terminal GOLD domain that extends into the lumen where it is thought to interact with cargo proteins (Dominguez et al., 1998; Kuehn et al., 1998). The second group of

GOLD domain proteins, which includes the yeast Osh3p, the human Golgi protein GCP60, and some animal Sec14-like proteins, are peripheral proteins that interact with membranes through a second type of interaction domain (e.g. FYVE, Sec14, PH). These proteins may act as adaptors for recruiting GOLD-domain binding proteins to specific internal membrane sites (Anantharaman and Aravind, 2002). PATL1, which belongs to this second group, may bind to the cell-plate membrane through its Sec14 domain and then recruit other proteins required for the later stages of cytokinesis through interactions with its C-terminal GOLD and variable N-terminal domains. Identification of proteins that bind to these domains will likely provide valuable clues to PATL1's mode of action.

Our intracellular localization studies are consistent with a model in which PATL1 is recruited to the division plane at the time of cell-plate expansion. In *Arabidopsis* root cells, PATL1 staining was most pronounced at expanding cell plates, as identified by the ring of phragmoplast microtubules (Fig. 4C). This timing corresponds to Phase III of the somatic cell cytokinesis model recently proposed by Segui-Simarro et al. (2004). During Phase III, vesicle traffic continues at the periphery while the central region of the cell plate begins consolidation and maturation. Our studies of tobacco BY2 cells, which afforded the opportunity to more precisely pinpoint the appearance of the PATL antigen at the cell plate, yielded similar results (Fig. 4, G–I). Detection of both a cytosolic and peripheral membrane-associated pool of PATL1 in biochemical fractionation studies was also consistent with the recruitment of PATL1 from the cytosol to the membranes of the developing plate (Fig. 3). The exact nature of the interaction of PATL1 with the membrane, however, is unclear at this time. The PATL1 sequence does not contain myristoylation, prenylation, or obvious palmitoylation signals, suggesting that the protein is not attached to the membrane by a posttranslationally added lipid anchor. Further experimentation will be required to determine if PATL1 interacts directly with membrane phospholipids or through interactions with other membrane proteins.

A comparison of our results to localization studies of other cell-plate-associated proteins is also consistent with a role for PATL1 in completion and maturation of the cell plate, rather than its initial assembly. Our localization results are very similar to those reported for ADL1A and E, a pair of dynamin-like proteins that are functionally redundant with respect to cell-plate biogenesis and maintenance of plasma membrane integrity (Kang et al., 2001, 2003). ADL1A and E localize to the expanding cell plate and, like PATL1, persist there for a time upon completion of cytokinesis (Kang et al., 2003). Similar localization patterns were also reported for a plant myosin VIII, which is thought to be involved in the development of plasmodesmata during maturation of the cell plate (Reichelt et al., 1999). Our results are in marked contrast to those reported for proteins involved in early membrane-trafficking

events such as translocation of vesicles to the division plane and vesicle fusion. AtPAKRP2, the kinesin-related protein thought to drive Golgi-derived vesicles to the plate, localizes to punctate structures that first appear at the division plane in early anaphase (Lee et al., 2001). Plate-associated PATL1 was not detected at this time. KNOLLE, the syntaxin that is required for early vesicle fusion events at the plate, associates with the plate in early telophase (Lauber et al., 1997) also before we first detected plate-associated PATL1. Furthermore, during cell-plate expansion both AtPAKRP2 and KNOLLE are most abundant at the periphery of the expanding plate, the site of continued vesicle addition, in contrast to PATL1, which was more concentrated in the central regions of the plate that are undergoing consolidation and maturation.

Although the majority of vesicle traffic is completed by Phase III, it continues to be important during the completion of cytokinesis (Segui-Simarro et al., 2004). The addition of vesicles to the periphery of the expanding plate continues along the remaining phragmoplast microtubules, although PATL1's distribution across the plate argues against its involvement in this activity (Fig. 4, J–L). PATL1 is more likely to function in other membrane-trafficking events that are occurring at this time. After fusion of the cell plate with the parent plasma membrane, vesicle traffic is required to close the remaining discontinuities in the membranous network. Membrane recycling from the membranous network stages is also extensive at this time, and the presence of clathrin-coated vesicles and endocytic multivesicular bodies in close proximity suggest that at least some fraction of this is clathrin dependent (Samuels et al., 1995; Battey et al., 1999; Otegui et al., 2001; Segui-Simarro et al., 2004). PATL1's localization at the cell plate, at a time of clathrin-dependent membrane recycling, is especially interesting in light of the presence of a motif (YGEFQ) that is recognized by  $\gamma$ -ear domains of clathrin-adaptor proteins (Mattera et al., 2004).

While, to the best of our knowledge, this is the first report to provide evidence for the involvement of a SEC14-like protein in cytokinesis in plant cells, there is evidence that proteins related to Sec14p in structure and/or function play a role in membrane traffic during cytokinesis in other organisms. Spo20, a *Schizosaccharomyces pombe* homolog of Sec14p, is required for the completion of cytokinesis in vegetative cells and shows defects in completion of septation (Nakase et al., 2001). In wild-type cells, the spo20 protein localizes in an actin-dependent fashion to growing cell tips and the medial septation site, both areas of active membrane construction. Nir2, a multidomain mammalian protein that belongs to the animal-specific group of PITPs, is also essential for the completion of cytokinesis (Litvak et al., 2002; Tian et al., 2002). Nir2 is recruited from the cytoplasm to the midbody, a microtubule-based structure that is critical for cleavage furrow ingression and membrane trafficking to the site of cell cleavage. Although the exact roles of Spo20

and Nir2 in cytokinesis are unclear, a likely possibility is that their PtdIns transfer/binding domains function in some way in vesicle traffic to the cleavage plane during cytokinesis.

Lipid-binding studies confirmed our sequence-based prediction that PATL1 would bind phospholipids (Fig. 5). PATL1's preference for PtdIns and its phosphorylated derivatives, PtdIns(5)P, PtdIns(4,5)P<sub>2</sub>, and PtdIns(3)P, distinguish it from Sec14p, which preferentially binds to PtdCh and PtdIns. Conservation of key residues in the PATL1 Sec14 lipid-binding pocket suggests that this domain may bind phosphoinositides (Fig. 1B). Furthermore, there are several examples of proteins in which Sec14 domains serve as phosphoinositide-binding modules. The soybean Sec14-like proteins, Ssh1p and Ssh2p, both bind PtdIns(4,5)P<sub>2</sub> with high affinity (Kearns et al., 1998b), and the human MEG protein tyrosine phosphatase, which regulates secretory vesicle fusion in T cells, binds PtdIns(3,4,5)P<sub>3</sub>, specifically via its Sec14 domain (Huynh et al., 2003). The PATL1 C-terminal Lys-rich motif, which is reminiscent of sequences found in AP180 and  $\mu$ 2-adaptin, is also an excellent candidate for a phosphoinositide-binding site (Ford et al., 2001; Mao et al., 2001; Rohde et al., 2002). Similar positively charged phosphoinositide-binding sites are found in the actin-binding proteins villin, gelsolin, and profilin (Janmey et al., 1992; Raghunathan et al., 1992; Yu et al., 1992; Sohn et al., 1995), synaptotagmin (Fukuda et al., 1995), and several clathrin-adaptor proteins (Gaidarov and Keen, 1999; Ford et al., 2001; Mao et al., 2001; Rohde et al., 2002). An important goal of future studies will be to determine if both the Sec14 domain and the C-terminal Lys-rich motif are functional lipid-binding sites and, if so, their specificity.

PATL1's ability to bind specific phosphoinositides and its localization at the cell plate suggest a role for these lipids in cell-plate biogenesis. Such a role is not surprising given the importance of phosphoinositides, particularly PtdIns(4,5)P<sub>2</sub> and PtdIns(3)P, in regulating membrane traffic (Czech, 2000; Huijbregts et al., 2000; Cullen et al., 2001; Bankaitis and Morris, 2003; Gruenberg, 2003). Phosphoinositides typically exert their influence by directly regulating the activity of their target proteins upon binding to PtdIns-specific domains or by recruiting and assembling protein complexes to specific lipid microdomains. The phosphoinositide-binding activity of PATL1 may account for its recruitment to the cell plate, perhaps to a specific lipid microdomain. Once there, it could recruit other proteins involved in vesicle formation via its GOLD or N-terminal domains.

PATL1 may also function to establish phosphoinositide microdomains, required for vesicle formation during the late stages of cytokinesis, through regulation of phosphoinositide synthesis. PITPs of both the Sec14 and animal-specific families stimulate phosphoinositide synthesis. In fact, Sec14p and animal PITPs are interchangeable in the phosphoinositide synthesis-dependent reconstitution of secretion (Hay

et al., 1995; Ohashi et al., 1995; Jones et al., 1998) and phospholipase C-signaling pathways in permeabilized mammalian cells (Cunningham et al., 1995, 1996; Kauffmann-Zeh et al., 1995). Although the mechanism is unknown, PITPs most likely stimulate phosphoinositide synthesis by serving as cofactors for PtdIns kinases. PITPs stimulate the activity of PtdIns kinases in vitro (Panaretou et al., 1997; Jones et al., 1998; Monks et al., 2001), and in some cases there is evidence that PITPs and PtdIns kinases are physically associated as well (Kauffmann-Zeh et al., 1995; Panaretou et al., 1997; Aikawa et al., 1999).

In conclusion, our results provide evidence that PATL1 is a novel phosphoinositide-binding protein that plays a role in membrane traffic during the expansion and maturation stages of cytokinesis. The presence of a family of related proteins in the Arabidopsis genome indicates that there may be redundancy of function, a likelihood if PATL1 plays an essential role in development of the cell plate. Sequence divergence, which is most pronounced in the N termini, may reflect specialization of function as well. The variable N-terminal domains are likely to confer upon each member of the family the ability to interact with different partners in the cell or to be recruited to distinct locations. Identification of binding partners and the characterization of PATL mutants is certain to lead to exciting advances in our understanding of the cellular functions of this novel group of proteins.

## MATERIALS AND METHODS

### Growth of Plant Material

Arabidopsis ecotype Columbia plants were grown in 1× Murashige and Skoog salts plus vitamins and 1% (w/v) Suc in liquid culture or on plates solidified with 0.7% plant tissue culture agar (Carolina Biological Supply, Burlington, NC). Seedlings were grown at 22°C with a 16-/8-h photoperiod or in continuous light with 150  $\mu$ mol photon m<sup>-2</sup> s<sup>-1</sup>. Tobacco Bright-Yellow 2 (BY2) cells were grown in suspension culture in modified Linsmaier-Skoog medium as described (Nagata et al., 1981).

### PATL1 Cloning

PATL1 was identified from a tryptic peptide (ALQLFQDNYPEF) of a protein that copurified with a zucchini (*Cucurbita pepo*) membrane-associated F-actin-binding protein that will be described elsewhere. A BLAST search at the time identified four Arabidopsis EST sequences corresponding to two different mRNAs, now known to be PATL1 and PATL5 (Altschul et al., 1990). A full-length PATL1 cDNA was produced by 5'- and 3'-RACE PCR using gene-specific primers designed from the PATL1 EST sequences (Marathon-Ready cDNA kit, CLONTECH, Palo Alto, CA).

### Sequence Analysis

BLAST algorithms were used to retrieve PATL sequences from public databases at NCBI (<http://www.ncbi.nlm.nih.gov>; Altschul et al., 1990). Alignments were performed using ClustalW (gap opening penalty = 10; gap extension penalty = 0.2; Thompson et al., 1994) and prepared for publication using ESPript (<http://prodes.toulouse.inra.fr/ESPrpt/cgi-bin/ESPrpt.cgi>). Domain analysis of the PATLs was performed with InterProScan (<http://www.ebi.ac.uk/InterProScan/>). The endpoints of the Sec14 domain of

the PATLs were defined by pairwise alignments of the PATL sequences with yeast (*Saccharomyces cerevisiae*) Sec14p (GenBank accession no. P24280). GOLD domains (Prosite:PDOCS0866) were identified by ScanProsite (<http://ca.expasy.org/tools/scanprosite/>; Gattiker et al., 2002). The position of potential coiled coils within the variable N-terminal domains was predicted by the Coils2 and Paircoil programs (Lupas et al., 1991; Berger et al., 1995).

### PATL1 Antiserum Production

Sequences coding for full-length PATL1 and 2 as well as the GOLD domain of PATL1 (amino acid nos. 383–573) were cloned into the GST-fusion expression vector pGEX6P-1 (Amersham Biosciences). Inserts were produced by PCR using full-length cDNA as template and primers that created *Bam*H1 and *Sal*I sites at the 5' and 3' ends, respectively. The resulting constructs were verified by DNA sequence analysis before transformation into *Escherichia coli* BL21 pLysS cells for protein production. Protein production was induced in early log phase cultures by the addition of 0.5 mM isopropyl- $\beta$ -D-thiogalactoside for 3 to 4 h at 37°C. The cells were harvested, washed with cold phosphate-buffered saline (PBS), and lysed in 1% (v/v) Triton X-100, 0.2 M NaCl, 20 mM sodium phosphate, pH 7.5, 5 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride. The lysate was cleared by centrifugation at 27,000g for 30 min and then incubated with GST-Sepharose beads (Amersham Biosciences) for 4 to 24 h. The beads were washed extensively with PBS and eluted with 20 mM reduced glutathione, 150 mM NaCl, and 100 mM Tris-Cl, pH 8. Purified PATL1-GST fusion proteins were cleaved with PreScission Protease according to the manufacturer's instructions (Amersham Biosciences). GST-Sepharose chromatography was used to remove the free GST. Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Protein preparations were analyzed by SDS-PAGE followed by staining with SYPRO Red (Molecular Probes, Eugene, OR) and scanning with a Storm 860 fluorescence scanner (Amersham Biosciences). The purified PATL1 proteins were injected into rabbits for antiserum production.

### Immunoblot Analysis

Arabidopsis organs, from seedlings grown on plates with a 16-/8-h photoperiod, were powdered in liquid N<sub>2</sub> and stored at -80°C. Total proteins extracts were prepared by vortexing a sample of tissue powder in ice-cold extraction buffer (0.125 M Tris-Cl, pH 6.8, 4% [w/v] SDS, 1 mM DTT, and 10% [v/v] glycerol). The extracted proteins were denatured at 95°C for 15 min and then microfuged for 10 min at room temperature. Total protein concentration of the cleared extract was determined using the bicinchoninic acid assay. Proteins were separated by SDS-PAGE and transferred to Immobilon-P 0.45  $\mu$ m polyvinylidene difluoride membranes (Millipore, Bedford, MA) by electroblotting. Blots were blocked for 1 h in 5% (w/v) powdered milk in Tris-buffered saline plus Tween 20 (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.05% [v/v] Tween 20) and then probed with antibody diluted in blocking solution for 1 to 4 h. Primary antibodies were diluted as follows: anti-PATL1, 1:5,000 to 1:10,000; anti-SEC12, 1:500 (Secant Chemicals, Winchendon, MA), and C4 mouse anti-actin, 1:400 (ICN, Aurora, IL). The blots were then washed in Tris-buffered saline plus Tween 20 and probed with Cy5- or Alexa488-goat anti-rabbit secondary antibodies (2  $\mu$ g/mL) for 1 h (Molecular Probes). The blots were washed and then scanned with a STORM 860 fluorescence scanner.

### Biochemical Fractionation

Arabidopsis roots, from plants grown in liquid culture with continuous light, were powdered in liquid N<sub>2</sub> and stored at -80°C. Powdered roots were homogenized using a mortar and pestle in 3 to 4 volumes of ice-cold homogenization buffer (25 mM Tris-Cl, pH 7.2, 0.25 M Suc, 3 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and one complete protease inhibitor tablet/50 mL; Roche Applied Science, Indianapolis). The homogenate was filtered through a single layer of Miracloth (Calbiochem, San Diego) and centrifuged at 1,000g to remove nuclei and debris. The resulting S1 fraction was ultracentrifuged at 100,000g for 2 h at 4°C to yield cytosolic (S100) and microsomal membrane fractions (P100). The pellets (P100) were resuspended in homogenization buffer. Tissue equivalents of S1, S100, and P100 fractions were subjected to immunoblot analysis as described above. To determine the nature of PATL1's interaction with the membrane, samples of the P100 fraction were pelleted at 100,000g for 1 h, resuspended in homogenization buffer that contained 1 M NaCl, 2 M urea, 1% (v/v) Triton X-100, or 1% (w/v) SDS, and incubated on ice for 1 h. An aliquot was set aside for a total

protein sample (T). The remainder was centrifuged at 100,000g for 1 h to produce the supernatant or solubilized protein fraction (S). S and T fractions were analyzed on immunoblots.

### Immunofluorescence Microscopy

Immunostaining of Arabidopsis roots, grown on vertical plates or in liquid culture with continuous light, was as described (Harper et al., 1996). PATL1 antibody was diluted 1:250 to 1:500. Microtubules were stained using rat anti- $\alpha$  tubulin monoclonal YOL1/34 (1:100; Accurate Chemical and Scientific Corporation, Westbury, NY). TO-PRO-3, diluted 1:1,000 in the last 10 to 20 min of the final washes, was used as a nuclear counterstain (Molecular Probes). Alexa 488 and 568 goat secondary antibodies (2  $\mu$ g/mL) were used to detect PATL1 and tubulin, respectively.

Early log phase BY2 cell cultures (wild type or the GmMan1::GFP line of Nebenfuhr et al., 1999) were transferred to polyethyleneimine-coated coverslips and allowed to settle for 10 min. The culture medium was removed, and the cells were prefixed for 15 min with 0.4 mM 3-maleimidobenzoyl-N-hydroxy-succinimide ester; Calbiochem) in 50 mM PIPES, 2 mM MgSO<sub>4</sub>, and 5 mM EGTA, pH 6.8 (1  $\times$  PME), followed by fixation with 4% (w/v) paraformaldehyde, 0.1% (w/v) glutaraldehyde, 1 $\times$  PME, 0.05% (v/v) Triton X-100, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride for 1 h. After washing for 3  $\times$  10 min with PBS (2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 13.69 mM NaCl, and 0.81 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4), the cell walls were digested for 2 to 3 min with 1 $\times$  PME, 0.5% (w/v) bovine serum albumin, 0.01% (w/v) cellulase (Onozuka, Tokyo), 0.001% (w/v) Y23 pectolyase (Kikkoman, Tokyo), and 0.5% (v/v) Triton X-100. The coverslips were then submerged in -20°C methanol for 15 min. After air drying, the cells were rehydrated in PBS, blocked at reverse transcription for 1 h in antibody buffer (1% [w/v] bovine serum albumin in PBS with 0.05% [w/v] azide), incubated with primary antibodies in antibody buffer overnight at room temperature, and washed with PBS. The cells were incubated for 2 to 4 h with secondary antibodies, diluted as described above, in antibody buffer. Finally the cells were washed with PBS and counterstained with TO-PRO-3, as described above.

Images were collected using a Leica (Wetzlar, Germany) TCS NT/SP confocal microscope equipped with HC PL APO 20 $\times$ /0.7, HCX PL APO 40 $\times$ /1.25 to .75, and PL APO 100 $\times$ /1.4 to .7 oil immersion objective lenses. Excitation was from argon (488 nm), krypton (568), and HeNe (633 nm) lasers. For triple labeling experiments, sequential scans were collected at each wavelength. The average size of the cytoplasmic puncta was determined using C-Imaging software (Compix, Cranberry Township, PA).

### Vesicle-Binding Assays

Natural egg PtdEth, soybean (*Glycine max*) PtdCh, and PtdIns in chloroform were obtained from Avanti Polar Lipids (Alabaster, AL). Synthetic PtdIns and phosphoinositides (with 16 C chains) were obtained as lyophilized powders from Echelon Laboratories (Salt Lake City). Unilamellar vesicles were produced by multiple extrusions through a 0.1- $\mu$ m polycarbonate filter using a LiposoFast microextruder (Avestin, Ottawa) as previously described (MacDonald et al., 1991). Recombinant PATL1 protein was precleared by spinning at 164,000g for 30 min in an Airfuge (Beckman Instruments, Palo Alto, CA) to remove insoluble protein. For binding experiments, precleared PATL1 (2.5  $\mu$ g) was mixed with vesicles in lipid buffer (0.15 M NaCl, 10 mM MOPS, pH 7, 0.1 mM EDTA). The samples were incubated at room temperature for 1 h and centrifuged at 164,000g for 30 min to pellet the vesicles along with any bound protein. Equivalent amounts of supernatant (S) and pellet (P) were analyzed by SDS-PAGE gels stained with SYPRO Red. PATL1 was quantified using the ImageQuant software package (Amersham Biosciences).

### 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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