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Arabidopsis \textit{EXO70A1} recruits Patellin3 to the cell membrane independent of its role as an exocyst subunit \textsuperscript{FA}

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Abstract The exocyst is a well-known complex which tethers vesicles at the cell membrane before fusion. Whether an individual subunit can execute a unique function is largely unknown. Using yeast-two-hybrid (Y2H) analysis, we found that \textit{EXO70A1} interacted with the GOLD domain of Patellin3 (PATL3). The direct \textit{EXO70A1}-PATL3 interaction was supported by in vitro and in vivo experiments. In \textit{Arabidopsis}, \textit{PATL3-GFP} colocalized with \textit{EXO70A1} predominantly at the cell membrane, and \textit{PATL3} localization was insensitive to BFA and TryA23. Remarkably, in the \textit{exo70a1} mutant, \textit{PATL3} proteins accumulated as punctate structures within the cytosol, which did not colocalize with several endomembrane compartment markers, and was insensitive to BFA. Furthermore, \textit{PATL3} localization was not changed in the \textit{exo70e2}, \textit{PRsec6} or \textit{exo84b} mutants. These data suggested that \textit{EXO70A1}, but not other exocyst subunits, was responsible for \textit{PATL3} localization, which is independent of its role in secretory/recycling vesicle-tethering/fusion. Both \textit{EXO70A1} and \textit{PATL3} were shown to bind \textit{PI4P} and \textit{PI(4,5)P}\textsubscript{2}, in vitro. Evidence was obtained that the other four members of the \textit{PATL} family bound to \textit{EXO70A1} as well, and shared a similar localization pattern as \textit{PATL3}. These findings offered new insights into exocyst subunit-specific function, and provided data and tools for further characterization of \textit{PATL} family proteins.

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INTRODUCTION

Across the eukaryotes, the exocyst complex consists of eight subunits, i.e., \textit{SEC3}, \textit{SEC5}, \textit{SEC6}, \textit{SEC8}, \textit{SEC10}, \textit{SEC15}, \textit{EXO70}, and \textit{EXO84}, and is thought to tether the secretory vesicles at the target membranes pending SNARE-mediated fusion (He and Guo 2009). In yeast and mammalian cells, each exocyst subunit is encoded by a single gene (Lipschutz and Mostov 2002). In \textit{Arabidopsis}, however, except for \textit{SEC6} and \textit{SEC8}, exocyst subunits are encoded by multiple genes. For example, there are 23 \textit{EXO70} paralogs (Synek et al. 2006; Cvrckova et al. 2012), which is suggestive of their diverse functions.

\textit{EXO70A1} has been implicated in plant cytokinesis (Fendrych et al. 2010). Cytokinesis is the final step of cell division, partitioning the cytoplasm between two sets of daughter chromosomes. Beginning at late anaphase, vesicles containing cell wall material, membrane lipids and proteins are initially delivered to the center of the division plane to form the nascent cell plate, which centrifugally expands and eventually fuses with the parental cell (Jürgens 2005a, 2005b; McMichael and Bednarek 2013). \textit{EXO70A1} was localized to the separation sites during initiation and maturation of the cell plate, when vesicle fusion is a frequent event (Fendrych et al. 2010). Consistently, \textit{exo70a1} displayed cytokinesis defects that were also observed in the \textit{exo84b}, and pollen-rescued \textit{sec6} homozygous mutants (PRsec6) (Fendrych et al. 2010; Wu et al. 2013; Rybak et al. 2014). Moreover, the accumulation of vesicles and large
membrane-bound compartments have been observed in exo84b and exo70a1 mutants, respectively (Fendrych et al. 2010; Li et al. 2013). Using variable-angle epifluorescence microscopy it was shown that the incidence of SEC6-GFP foci at the plasma membrane (PM) and the trafficking of VAMP721 (a marker of exocytosis in plants) to the PM was severely affected in the exo70a1 mutant (Fendrych et al. 2013). Taken together, these findings suggest that EXO70A1 functions together with other exocyst subunits, in vivo, to tether vesicles to the cell plate during cytokinesis (Zarsky et al. 2013).

Besides cytokinesis, EXO70A1 also plays joint roles with various exocyst subunits in hypocotyl and root hair elongation (Synek et al. 2006; Hala et al. 2008), polarized seed coat pectin deposition (Kulich et al. 2010), integral PM protein (PINs) recycling and polar auxin transport (Drdova et al. 2013), as well as root meristem size and root cell elongation (Cole et al. 2014). Moreover, EXO70A1 cofractions with SEC3, SEC5, SEC6, SEC8 as the core of the complex, and interacts with several exocyst subunits in Y2H assays (Hala et al. 2008). It is therefore considered that EXO70A1 might execute its diverse physiological functions within the exocyst complex (Hala et al. 2008; Zarsky et al. 2013). Whether EXO70A1 has a unique, exocyst-independent role is not known.

Here, we report that EXO70A1 interacts, in vitro and in vivo, with PATL3. PATL proteins bear an additional GOLD-domain other than the SEC14-domain, and are related to SEC14 proteins that are implicated in membrane trafficking, cytoskeleton dynamics, lipid metabolism and lipid-mediated regulatory functions (Phillips et al. 2006; Bankaitis et al. 2007; Mousley et al. 2007). Arabidopsis PATL family consists of six members (PATL1-6). PATL1 and PATL2 show different binding affinity for phospholipids, and localize to the cell plate during cytokinesis (Peterman et al. 2004; Suzuki et al. 2016), whereas PATL3 and PATL6 play a role in inhibiting Alfalfa mosaic virus movement (Peiro et al. 2014). We found that PATL3 predominantly localized to the PM and cell plate, coinciding with its initiation, expansion and maturation, while this localization was not achieved via the secretory or endocytic pathways. Most strikingly, the targeting of PATL3 to the cell membrane was shown to be EXO70A1-dependent, and independent of SEC6, EXO84b or EXO70E2. We conclude that EXO70A1 plays a subunit-specific role in recruiting PATL3 by direct binding, and propose that this may involve binding to the signaling lipids, phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2). There are six PATLs in Arabidopsis (Peterman et al. 2004; Ghosh and Bankaitis, 2011) and we show that they also share a similar localization pattern and targeting mechanism.

**RESULTS**

**Identification of PATL3 as an EXO70A1-binding protein**

Previous studies revealed that EXO70A1 is involved in cell polarity and morphogenesis, cell division, cell wall biogenesis, integral PM protein recycling and polarization (Hala et al. 2008; Fendrych et al. 2010; Kulich et al. 2010; Drdova et al. 2013), but the exact molecular mechanism awaits to be explored. To further investigate the roles of EXO70A1, a yeast Arabidopsis cDNA library was screened using full-length EXO70A1 as bait. Approximately 1 x 10⁷ yeast transformants were screened on a quadruple dropout (QDO) medium supplemented with X-α-gal. Two positive clones identified corresponded to the S1 + G domain of PATL3 as defined below (Figure 2A).

The interaction detected in Y2H was further verified by different approaches. In a glutathione S-transferase (GST) pull-down assay, EXO70A1-GST proteins were able to pull down PATL3-MBP (maltose binding protein) but not MBP, whereas GST alone could not pull down either prey (Figure 1B). To perform a coimmunoprecipitation (CoIP) assay, rabbit anti-EXO70A1 antibodies were first generated and were able to recognize EXO70A1 proteins specifically (Figure 1C, left panel). The lysate of pro35S:PATL3-GFP plants was incubated with GFP-Trap, and the CoIP result showed that the endogenous EXO70A1 proteins could be immunoprecipitated, together with PATL3-GFP (Figure 1C, right panel). In a firefly luciferase complementation imaging (LCI) assay (Chen et al. 2008), EXO70A1-HA was fused to the C-terminal domain of luciferase (cLUC), while Flag-PATL3 was fused to the N-terminal domain (nLUC), and expression of the fusion proteins in infiltrated tobacco leaves was confirmed (Figure S1). These results showed that only co-expression of EXO70A1-HA-cLUC and Flag-PATL3-NLUC in tobacco leaves could reconstitute a high
luciferase activity, compared to the various negative controls (Figure 1D). Taken together, these results demonstrated that EXO70A1 was able to interact with PATL3, both in vitro and in vivo.

EXO70A1 interacts with the C-terminal GOLD domain of PATL3

PATL-family proteins contain a variable N-terminal (N) region, a conserved Sec14 lipid-binding (S) domain, and a GOLD (G) domain (Figure 2A) (Peterman et al. 2004). A series of PATL3 truncations fused with the activation domain (AD) of GAL4 were generated in order to narrow down the region required for EXO70A1 binding (Figure 2A). The results showed that only PATL3 (S + G), (S1 + G) and (G) truncations, which contained at least the GOLD domain, could interact with EXO70A1 (Figure 2B), although full-length PATL3 was unable to interact with EXO70A1, probably because it could not be
expressed well in the nuclei or was not folded properly in yeast (Figure 2B). Next, a bimolecular fluorescence complementation (BiFC) assay (Ohad et al. 2007) was carried out using the same set of PATL3 truncations (Figure 2A) fused with the C-terminus of the split-yellow fluorescent protein (YFP<sup>C</sup>) and EXO70A1 fused with the N-terminus of YFP (YFP<sup>N</sup>). Bright fluorescent signals highlighted the PM when full-length PATL3-YFP<sup>C</sup> was co-infiltrated with EXO70A1-YFP<sup>N</sup> (Figure 2C). Only signals diffused in the cytosol and on the plasmodesmata-like structures were detected when each truncation containing at least the GOLD domain fused to YFP<sup>C</sup> was co-transformed with EXO70A1-YFP<sup>N</sup> (Figure 2C). The expression of fusion proteins in the BiFC assays was confirmed (Figure S1). Collectively, these data indicated that the GOLD domain of PATL3 was the minimal region for EXO70A1 interaction, while the N-terminus might further assist their spreading on the PM.

The subcellular localization of PATL3

In non-dividing root meristematic cells of pro35S:PATL3-GFP plants, PATL3 was observed diffusing in the cytosol and labeling the PM (Figure 3, indicated by arrowheads). Interestingly, at the onset of cytokinesis (indicated by arrows), additional PATL3 signals appeared at the nascent cell plate, overlapping with FM4-64 fluorescence, and as a diffuse cloud around the cell equator (Figure 3; t = 0 min). At subsequent time points, PATL3 labeled the laterally expanding-cell plate, which finally fused with the parental PM (Figure 3). Transgenic Arabidopsis plants harboring ProPATL3:PATL3-GFP displayed the same localization pattern as with the 35S promoter (Figure S2), indicating that overexpression did not alter the localization of PATL3. Furthermore, in pro35S:PATL3-GFP transgenic BY-2 cells, PATL3 achieved a similar localization, and the PM association was confirmed by plasmolysis (Figure S3). Altogether, these results demonstrated that PATL3 was...
cell membrane-associated and recruited to the cell plate during cytokinesis.

**EXO70A1 colocalizes with PATL3 and is required for the localization of PATL3 to the cell membrane**

As EXO70A1 interacted with PATL3 (Figure 1), we next wanted to know how this interaction was spatially and temporally coordinated, at the cellular level. Therefore, pro35S:EXO70A1-mCherry was crossed with pro35S:PATL3-GFP to generate double-labeling lines and their localization patterns studied. As shown in Figure 4, PATL3 colocalized with EXO70A1 at both the PM (Figure 4A) and expanding cell plate (Figure 4B, C). To determine the significance of this colocalization, PATL3-GFP/PATL3-GFP exo70a1-2/− mutants were chosen through antibiotic selection and PCR-based genotyping (Figure S4). Compared to the PM and cell plate localization in wild-type cells (Figure 4A–C), PATL3 appeared as punctate structures in the cytoplasm of exo70a1-2/− non-dividing and dividing cells, which did not overlap with FM4-64 fluorescence (Figure 4D). This dislocation of PATL3 in the exo70a1-2/− mutant was observed in all root cells (Figure S5). These data indicated that the localization of PATL3 to the cell membrane was dependent on EXO70A1.

**PATL3 recruitment is not dependent on the secretory or endocytic pathway**

So far, EXO70A1 has always been suggested to function as a subunit of the exocyst complex, mediating the tethering of secretory/recycling vesicles to target membranes (Zarsky et al. 2013). Hence, it became interesting to investigate the nature of the PATL3 punctate structure in the exo70a1-2 mutant background. In summary, we found that these intracellular PATL3 punctate structures did not overlap with FM4-64 (Figure 5A), the Golgi marker SYP32-mRFP (Figure 5B; Gao et al. 2014), or the TGN marker SYP43-mCherry (Figure 5C; Uemura et al. 2004). After treatment with BFA, PATL3 aggregates remained distinct from BFA compartments (Figure 5D). Furthermore, after treatment with 100 μM BTH (Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester, a salicylic acid agonist that triggers the autophagic pathway in Arabidopsis) for 6 h, the autophagosome marker ATG8e-mCherry translocated from the cytosol to the punctate compartments, while PATL3 punctates remained unchanged (Figure 5E, F), indicating that, in exo70a1-2, they were not autophagosomes.

The possible trafficking pathways of PATL3-GFP were further explored. Using Arabidopsis seedlings treated with 50 μM BFA for 2 h and then with FM4-64 for 15 min, PIN2-GFP and FM4-64 were found to accumulate in BFA compartments, while EMP12-GFP labeled Golgi apparatus was found to surround them (Figure S6), as reported earlier (Richter et al. 2007; Kleine-Vehn et al. 2008a, 2008b; Gao et al. 2012). However, in pro35S:PATL3-GFP transgenic root cells, while FM4-64 accumulated in the BFA compartments,
PATL3-GFP localization remained unchanged (Figure 6A), even when it was further crossed into gnl1-1 (Figure S7), where Golgi-localized BFA-insensitive ARF-GEF GNL1 had been mutated (Figure 6B; Richter et al. 2007). In cells undergoing cytokinesis, YFP-RABA2a, necessary for the delivery of TGN-derived vesicles to the leading edge of the cell plate (Chow et al. 2008), accumulated in the BFA compartments, while the cell plate localization of PATL3 was not affected by BFA (Figure 6C). These results strongly suggested that PATL3 targeting was not mediated by the secretory pathway.

Previously, results established that endocytic delivery of cell surface material contributes to cell plate formation during plant cytokinesis (Dhonukshe et al. 2006). To investigate whether the cell plate-localized PATL3 was derived from the parental cell, the clathrin-mediated endocytosis inhibitor tyrphostin A23 (TyrA23) was used. In agreement with previous work, the localization of CLC-mOrange (Ito et al. 2012) was dislodged (Figure S6), and the internalization of FM4-64 was strongly inhibited (Figure 6D) after TyrA23 treatment. However, this drug had no visible effect on the cell plate targeting of PATL3-GFP (Figure 6D). Therefore, PATL3-GFP cell plate targeting was not depend on clathrin-mediated endocytosis.

Taken together, these results suggested that PATL3 recruitment to both cell membranes is distinct from the conventional secretory or endocytic pathway; rather, it should be recruited from the cytoplasm by directly binding to EXO70A1.

The recruitment of PATL3 is not dependent on EXO70E2, SEC6 or EXO84b

PATL3 was detected on uncharacterized punctate structures in the exo70ai mutant (Figures 4, 5), and its cell membrane targeting was shown to be independent of the secretory or endocytic pathway (Figure 6). These data strongly suggested that PATL3 proteins are not packed into the vesicles, and that its recruitment might not be due to the vesicle-tethering function of the exocyst complex.

To test these notions, pro35S:PATL3-GFP was introduced into the PRsec6 and exo84b mutants, and the localization pattern of PATL3 in these mutants was further explored. Like EXO70A1, both SEC6 and EXO84b localized to the cell plate, confirming their role in cytokinesis (Fendrych et al. 2010; Wu et al. 2013; Rybak et al. 2014). Our previous study defined PRsec6 as homozygotes of the sec6/SEC6 pLAT52:SEC6/pLAT52:SEC6 mutant progenies, and PRsec6-1 was shown to be a null mutant with no SEC6 protein expression (Wu et al. 2013). SEC6 is a single copy gene in Arabidopsis, thus, in the PRsec6 mutant the exocyst holo-complex should be absent. In the exo84 mutant, vesicle accumulation was observed, suggestive of a defect in vesicle tethering (Fendrych et al. 2010). EXO70E2 defines the Exocyst-positive Organelle (EXPO), which is involved in the unconventional protein secretion pathway (Wang et al. 2010; Ding et al. 2012, 2014). Our results showed that membrane recruitment of PATL3 was only perturbed in the exo70ai mutant, and not in the PRsec6, exo84b and exo70e2 homozygous mutants (Figures 7, S7; Wu et al. 2016).
These data suggested that, in addition to functioning in vesicle tethering, EXO70A1 plays a unique, exocyst-independent role in PATL3 recruitment.

PATL3 and EXO70A1 bind phosphoinositides

In the budding yeast *Saccharomyces cerevisiae*, SEC3 and EXO70 are thought to target to the budding site by binding to the minor lipid, PI(4,5)P₂, which serves as a landmark for assembly of the complete exocyst complex (Boyd et al. 2004; He et al. 2007; Zhang et al. 2008). Arabidopsis PATL1 has also been shown to bind PI(4,5)P₂ and several other phosphoinositides (Peterman et al. 2004). EXO70A1-GST, PATL3-MBP and PATL3 SEC14 domain-MBP (SEC14-MBP) fusion proteins were made to test their phosphoinositide-binding capacity. All three proteins were found to bind PI(4,5)P₂, and its precursor, PI₄P, while GST or MBP alone did not (Figure 8A, B). The fusion proteins did not bind PA or PI₃P, two other negatively charged phospholipids that are involved in signaling and membrane binding (Vermeer et al. 2006, 2009; Munnik and Vermeer 2010; Munnik and Nielsen 2011; Testerink and Munnik 2011; Simon et al. 2014).

Figure 5. Characterization of PATL3 aggregates in the exo70a1-2 homozygous mutant

(A) The seedlings were stained with FM4-64 for 15 min. (B) and (C) The PATL3 aggregates were not overlapped with the Golgi marker SYP32-mRFP and the TGN marker SYP43-mCherry. (D) The PATL3 aggregates were insensitive to BFA. The seedlings were treated with 50 µM BFA for 2 h followed by FM4-64 for 15 min. (E) and (F) PATL3 aggregates were insensitive to the treatment of 100 µM BTH for 6 h. ATG8e-mCherry was used as a positive control. Bars = 10 µm (A, D, E, F), and 5 µm (B, C).

PATLs may be recruited in a similar way and involved in cytokinesis

PATL family proteins share approximately 45% overall sequence identity at the amino acid level. We were therefore curious whether other PATLs would also interact with EXO70A1, and localize to the membrane. Four additional PATLs (PATL1, PATL2, PATL4 and PATL6) were studied, except for PATL5, whose expression was barely detected by RT-PCR experiments and by publicly available microarray data (Figure S8). Using the BiFC assays, we showed that EXO70A1 also interacted with the GOLD domain of the additional four PATLs (Figure 9A). Moreover, in transgenic BY-2 cells, these PATL proteins exhibited similar subcellular localization patterns as PATL3, which was mainly localized to the PM with a weak cytosolic appearance in non-dividing cells, and to the cell plate in dividing cells (Figure 9B, C). The latter is also consistent with previous studies on the localization of PATL1 and PATL2 (Peterman et al. 2004; Suzuki et al. 2016). Thus, it seems that PATLs shared a similar strategy to achieve membrane localization and be involved in cytokinesis.
DISCUSSION

In this study, we report the characterization of an interaction between EXO70A1 and PATL3, and suggest that EXO70A1 is essential to recruit PATL3 to the membrane by direct binding. These results provide a new example of the existence of an exocyst subunit-specific function in plants. Moreover, PATL3 binds to PI4P and PI(4,5)P2 which might further enhance its membrane association and its subcellular localization resembles more that of PI4P.

Targeting of PATL3 is mediated in an EXO70A1-dependent manner

We demonstrated that EXO70A1 is responsible for the recruitment of PATL3 from its cytoplasmic pool to the
membrane, via direct binding. This conclusion was based on the following evidence: First, PATL3 was identified as an interacting protein of EXO70A1 by a Y2H screening, which was further confirmed by in vitro and in vivo interaction experiments (Figure 1). The interaction seems to be mediated by the GOLD domain of PATL3 (Figure 2). Second, EXO70A1 and PATL3 colocalized at the PM and the expanding cell plate in root meristematic cells (Figures 3, 4). Third and most importantly, when PATL3-GFP was introduced into an exo70a1 mutant background, its localization shifted from the cell membrane to punctate structures in the cytosol (Figure 4), which did not overlap with any of the endomembrane compartments tested and is BFA-insensitive (Figure 5). Indeed, PATL3 recruitment was found to be independent of the secretory and endocytic pathways (Figure 6). These PATL3-GFP punctate structures in exo70a1-2 could be protein–lipid aggregates. Our results strongly suggest that localization of PATL3 to the cell membrane is under strict regulation by EXO70A1. Furthermore, these data suggest that proteins recruited from the cytoplasm may contribute considerably to the cell plate formation, other than those delivered via secretory and endocytic pathways.

**Targeting of PATL3 to the cell membrane is mediated in an EXO70A1-specific manner**

Current models in mammalian and yeast cells suggest that the exocyst assembles into an octameric protein complex, and that in most cases all eight subunits are required for full exocytic function of the complex (TerBush et al. 1996; Hsu et al. 2004; Brennwald 2013). Likewise, the exocyst in Arabidopsis has been suggested to function as a complex (Hala et al. 2008; Fendrych et al. 2010). Compared to studies on the holo-complex, the cellular roles of exocyst-specific subunits are still limited. So far, only an EXO70-specific role has been shown in mediating the recruitment of the key actin polymerization factor, Arp2/3 complex and Nucleoporin Nup62, via direct interactions and regulating actin dynamics at the leading edges of migrating cells (Zuo et al. 2006; Hubert et al. 2009). The recruitment of PATL3 to the cell membrane was disrupted in exo70a1, but not in the PRsec6, exo84b and exo70e2 homozygous mutants (Figure 7), consistent with a plant exocyst subunit-specific function for EXO70A1, in addition to its vesicle tethering role within the exocyst complex.

**Possible natural phosphoinositide target of EXO70A1 and PATL3**

Protein–lipid overlay assays showed that both EXO70A1 and PATL3 bound phosphoinositides PI4P and PI(4,5)P2, but not PI3P or PA in vitro (Figure 8). Using lipid biosensors, PI4P has been shown to be constitutively present at the plasma membrane (Vermeer et al. 2009; Vermeer and Munnik 2013; Simon et al. 2014), and to accumulate on the expanding cell plate (Vermeer et al. 2009), just like PATL3. In contrast, there seems to be hardly any PI(4,5)P2 at the plasma membrane of plant cells, and was only found to accumulate at the leading edges of the cell plate just prior to fusing with the parental PM (van Leeuwen et al. 2007). Based on the in vivo
localization patterns, it is most likely that PI4P is the natural target of PATL3 and EXO70 at the cell membrane (van Leeuwen et al. 2007; Vermeer et al. 2009; Munnik et al. 2014). In vitro, PI(4,5)P2 did bind stronger but with three phosphates, this lipid is also slightly more negative than PI4P (two phosphates), which may account for the stronger binding. Moreover, Arabidopsis seedlings contain 20–30 times more PI4P than PI(4,5)P2 (Munnik et al. 1994; Munnik 2014). So both in vivo and in vitro data point to the participation of PI4P, even though it was not unlikely that whenever PI(4,5)P2 levels are boosted, for example, during cell plate extension (van Leeuwen et al. 2007), they could help attracting our target proteins to the membrane.

The functions of PATL family proteins might be fine-tuned

It is interesting to know that all PATLs studied here localize to the cell plate, which is consistent with earlier reports on PATL1 and PATL2 (Peterman et al. 2004; Suzuki et al. 2016), and we showed that this might occur by the same strategy, through interaction with EXO70A1 via their respective GOLD domains (Figure 9). Despite the similarities, differences were also noticed that could be indicative of additional control mechanisms other

Figure 9. PATLs may be recruited in a similar way and exhibit similar subcellular localization patterns as PATL3-GFP (A) EXO70A1 interacts with the GOLD domain of four additional PATLs by BiFC assay. (B, C) Subcellular localization of PATL-GFP fusion proteins in non-dividing (B) and dividing (C) transgenic BY-2 cells. Bars = 25 µm in (A), 20 µm in (B and C).
than by EXO70A1 recruitment. For example, PATL2 (Suzuki et al. 2016) and PATL3 are both detected from the onset of the cell plate until finally reaching the parental cell membrane; PATL1, however, only labels the cell plate at the time of its expansion and maturation, but not initiation (Peterman et al. 2004). Besides the differential cell plate localization patterns, the binding properties of PATL1-3 to phosphoinositides also appear to be distinct, even though PATLs share high similarity within their SEC14 domains (Peterman et al. 2004). PATL1 binds to PI and several phosphoinositides, including PI3P, PI4P, PI5P, PI(3,5)P and PI(4,5)P,

but has low affinity for PI(3,4)P2 (Peterman et al. 2004). In contrast, PATL2 binds to all these phosphoinositides, but has no affinity for PI (Suzuki et al. 2016). We found that PATL3 had no affinity for PI3P or PA but bound to PI4P and PI(4,5)P2 (Figure 8). It seems that all PATLs are involved in cytokinesis, but that their function is individually fine-tuned to integrate themselves into a distinct regulatory network. For example, PATL2 was identified as a substrate of mitogen-activated protein kinase (MAPK) cascade protein MAPK4 which is required for cytokinesis (Suzuki et al. 2016).

The gene expression patterns of PATLs were studied by β-glucuronidase (GUS) assays performed in transgenic lines harboring each of the five PATL promoters, respectively (proPATL1: 2,020 bp; proPATL2: 2,067 bp; proPATL3: 793bp; proPATL4: 1,970 bp; proPATL6: 1,988 bp in relation to ATG). Generally, these genes displayed similar ubiquitous expression patterns (Figure S9), which is consistent with microarray data (Figure S8). The precise role of PATLs in plant development, however, still remains unclear. Our data and tools should prove useful in further exploring the cellular functions played by these plant PATLs.

MATERIALS AND METHODS

Plant materials and growth conditions
Mutant lines ex070a1-2 (SALK_135462), ex084b-1 (GA-BI_459C01), ex070e2 (GK-072F12), and gnhl-1 (SALK_067415) were used in this study. Arabidopsis thaliana (Columbia-0) was used for transformation using floral dipping (Clough and Bent 1998). Plants were grown at 22°C under 16 h-light/8 h-dark conditions. BY-2 cells were cultured at 25°C on Murashige and Skoog (MS) medium (Sigma-Aldrich) in the dark and sub-cultured once a week for suspension cultures and twice a month for calli grown on agar plates.

Y2H screening
Y2H screening was performed using the MATCHMAKER GAL4 Two-Hybrid System, according to the supplier’s instructions (Clontech). Briefly, full-length EXO70A1 fused with the DNA-binding domain of GAL4 in pGBKT7 was used as a bait to screen an Arabidopsis cDNA library. The mated yeast cells were selected on QDO plates, and the putative positive clones grown on QDO were assayed for LacZ activity. Positive clones were further confirmed by cotransforming with pGBK7-EXO70A1 back into stain AH109. Colonies growing on QDO plates were further examined on selective plates containing Xα-Gal (40 μg/mL).

In vitro GST pull-down assays
Five micrograms each of GST and EXO70A1-GST fusion proteins were first immobilized on glutathione Sepharose 4B beads (GE Healthcare) in GST binding buffer (50 mmol/L Tris-HCl pH7.5, 1 mmol/L MgCl2, 1 mmol/L DTT), and then re-suspended in blocking buffer (400 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 1.8 mmol/L KH2PO4, 0.05%-0.1% NP-40, 0.5% non-fat milk) and gently rotated at 4°C for 1 h. Next, the beads were centrifuged and the blocking buffer discarded. The MBP or PATL3-MBP fusion proteins were added into the GST- and EXO70A1-GST- bound beads, respectively. The resulting solutions were re-suspended in binding buffer (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 1.8 mmol/L KH2PO4, 0.05%-0.1% NP-40) and incubated at 4°C for 1 h. Subsequently, the beads were washed with the wash buffer (400 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 1.8 mmol/L KH2PO4, 0.05%-0.1% NP-40) for five times. The samples were subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and the MBP and PATL3-MBP signals were detected by anti-MBP antibodies (Beijing Protein Innovation).

Agrobacterium infiltration and protein extraction of tobacco leaves
Agrobacterium strain GV3101 containing EXO70A1, PATL3 fusion constructs or p19 plasmid (Voinnet et al. 2003) was inoculated to 5 mL of LB medium, and grown at 28°C in a shaker for 48 h. The cells were transferred to fresh LB medium containing 10 mmol/L MES (pH 5.6)
and 40 \mu M acetosyringone (1:100 ratio, v/v). The bacterial cells were resuspended in 10 mmol/L MgCl₂ at a final O.D.₆₀₀ of 1.5. For the Agrobacterium strain containing p19, a final OD₆₀₀ of 1.0 was used. Acetosyringone at a final concentration of 200 \mu M was added to the Agrobacterium solution. For coinfiltration, an equal volume of Agrobacterium strain carrying different plasmid was mixed and infiltrated by depressing the plunger of a 1 mL disposable syringe to the abaxial side of 5- to 6-week old tobacco leaves. Three days after infiltration, tobacco leaves were ground in liquid nitrogen, and resuspended in ice-cold extraction buffer (50 mmol/L Tris-MES pH 8.0, 0.5 M sucrose, 1 mmol/L MgCl₂, 10 mmol/L EDTA, 5 mmol/L DTT, protease inhibitor cocktail (Roche)). The total extract was centrifuged at 16,000 g at 4°C for 30 min and the supernatant was used.

**EXO70A1 antibody production and co-immunoprecipitation assay**

Recombinant EXO70A1 in pCZnt plasmid was bacterially produced and purified. The protein was injected to the rabbits to generate polyclonal antibodies at Zoonbio Biotech Co., China. The EXO70A1 antibodies were used in 1:1,000 dilutions in immunoblot analyses.

Five grams of seedlings was ground in liquid nitrogen and suspended in lysis buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 0.5% [v/v] Triton X-100) supplemented with the protease inhibitors cocktail. After 30 min incubation on ice, cell debris was moved, and supernatants were incubated with GFP-trap (Chromotek) beads for 2 h at 4°C with gentle rotation. The beads were rinsed four times with washing buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 0.2% [v/v] Triton X-100) supplemented with the protease inhibitors cocktail. The samples were subject to SDS–PAGE, and the presence of the interacting partner was detected by anti-GFP or anti-EXO70A1 antibodies.

**Confocal microscopy**

Confocal images of fluorescent signals were collected using a LSM710 system (Zeiss, www.zeiss.com). GFP signals were excited at 488 nm and emission was detected at 505–530 nm. YFP were excited at 514 nm and emission was detected at 529–550 nm, mCherry were excited at 561 nm and emission was detected at 605–630 nm, FM4-64 were excited at 561 nm and emission was detected at 610–630 nm.

**Drug treatments**

For drug treatments, 4- to 7-d-old seedlings were transferred into 1/2 MS liquid medium containing different chemical and incubated for the time periods indicated. Drugs were used at the following concentrations: BFA (50 mmol/L in dimethylsulfoxide (DMSO), working solution 50 \mu M), Tyrphostin A23 and Tyrphostin A51 (30 mmol/L in DMSO, working solution 75 \mu M), FM4-64 (2 mmol/L in DMSO, working solution 4 \mu M). Each treatment was repeated at least three times.

**Protein-lipid binding assay**

Two methods were used to assay protein-lipid binding capacity. The filter binding was performed as described (Munnik and Wierzchowiecka 2013). In brief, synthetic dioleic-P(4,5)P₂, -Pi4P, -PA, and -Pi3P (Avanti Polar Lipids, AL, USA) were dissolved in chloroform/methanol/H₂O (20:9:1) and spotted onto nitrocellulose (Amersham Hybond-C Extra) in a concentration range from 2–256 pmol, followed by incubation with GST- or MBP-tag proteins. The filter was then incubated with anti-GST or anti-MBP antibodies. Proteins bound to lipids were visualized by chemical luminescence (ECL).

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AUTHOR CONTRIBUTIONS

Y.B. conceived and designed the research plans; C.W. performed most of the experiments; L.T., X.T., Y.L., Y.Z., B.L. and Q.R. provided technical assistance to C.W.; F.L. helped with photo processing; M.H. and T.M. carried out the protein-lipid interaction assays; C.W. and Y.B. designed the experiments and analyzed the data; C.W. and Y.B. wrote the paper.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article: [http://onlinelibrary.wiley.com/doi/10.1111/jipb.12578/suppinfo](http://onlinelibrary.wiley.com/doi/10.1111/jipb.12578/suppinfo)

**Figure S1.** Examination of protein expression of various constructs in the tobacco leaves for the LCI and the BiFC experiments

**Figure S2.** Transgenic *Arabidopsis* plants harboring ProPATL3:PATL3-GFP displayed the same localization pattern as that with 35S promoter

**Figure S3.** Subcellular localization of PATL3 in transgenic BY-2 cells

**Figure S4.** Genotyping of PATL3-GFP/PATL3-GFP exo70a1-2/exo70a1-2 mutant lines where all plants showed hygromycin resistant (selection marker for PATL3-GFP transgene)

**Figure S5.** The dislocation of PATL3-GFP in *exo70a1-2* mutant was observed in all root cells

Bars = 20 μm.

**Figure S6.** The positive or negative controls of BFA and TyrA23 treatments

**Figure S7.** Identification of PATL3-GFP in gnl1-1, *exo70e2*, *PRsec6-1* and *exo84b-1* homozygous mutant background

**Figure S8.** The expression pattern of the *Arabidopsis thaliana* PATL genes

**Figure S9.** Expression patterns of PATLs revealed in different proPATL:GUS transgenic *Arabidopsis*

**Table S1.** List of primer pairs used in this study