Adaptor Protein-3-Dependent Vacuolar Trafficking Involves a Subpopulation of COPII and HOPS Tethering Proteins

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Plant vacuoles are versatile organelles critical for plant growth and responses to environment. Vacuolar proteins are transported from the endoplasmic reticulum via multiple routes in plants. Two classic routes bear great similarity to other phyla with major regulators known, such as COPII and Rab5 GTPases. By contrast, vacuolar trafficking mediated by adaptor protein-3 (AP-3) or that independent of the Golgi has few recognized cargos and none of the regulators. In search of novel regulators for vacuolar trafficking routes and by using a fluorescence-based forward genetic screen, we demonstrated that the multispan transmembrane protein, Arabidopsis (Arabidopsis thaliana) PROTEIN S-ACYL TRANSFERASE10 (PAT10), is an AP-3-mediated vacuolar cargo. We show that the tonoplast targeting of PAT10 is mediated by the AP-3 complex but independent of the Rab5-mediated post-Golgi trafficking route. We also report that AP-3-mediated vacuolar trafficking involves a subpopulation of COPII and requires the vacuolar tethering complex HOPS. In addition, we have identified two novel mutant alleles of AP-3, whose point mutations interfered with the formation of the AP-3 complex as well as its membrane targeting. The results presented here shed new light on the vacuolar trafficking route mediated by AP-3 in plant cells.

Plant vacuoles are versatile organelles critical for plant growth and responses to the environment (Pacini et al., 2011). The functionality of vacuoles is fulfilled through proteins localized at the vacuolar membrane (i.e. the tonoplast) and those in the vacuolar lumen. Transmembrane proteins at the tonoplast mediate ion homeostasis and turgor regulation, whereas luminal proteins inside the vacuoles participate in nutrient recycling and signal termination.

Vacuolar proteins are synthesized at the endoplasmic reticulum (ER) and transported via multiple routes to their destination in plants (Bassham et al., 2008; Hwang, 2008; Pedrazzini et al., 2013; Uemura and Ueda, 2014). Four distinct vacuolar trafficking routes have been proposed in plant cells (Uemura and Ueda, 2014). A classic route bearing high similarity to yeast and metazoans utilizes COPII-mediated anterograde trafficking from the ER to the Golgi. It passes through the trans-Golgi network/early endosome (TGN/EE) to prevacuolar compartments (PVC)/multivesicular bodies (MVB) through sequential activation of the small GTPases Rab5 and Rab7 (Cui et al., 2014; Ebine et al., 2014; Singh et al., 2014) and finally reaches the vacuoles. The second route is slightly distinct from the first, such that Rab7 is not required (Ebine et al., 2014; Feng et al., 2017). The third route is mediated by Adaptor Protein-3 (AP-3), a heterotetrameric complex consisting of two large subunits (δ and β), a medium subunit (μ), and a small subunit (σ; Bassham et al., 2008; Feraru et al., 2010; Zwiewka et al., 2011). Finally, several vacuolar proteins have been reported to target to the tonoplast independent of the Golgi (i.e. directly from the ER to the vacuoles; Viotti et al., 2013). Many cargos and regulators have been identified for the first two routes (Bassham et al., 2008; Hwang, 2008; Pedrazzini et al., 2013; Uemura and Ueda, 2014). By contrast, few cargos have been identified for the AP-3-mediated trafficking route and the ER-to-vacuole direct route (Wolfenstetter et al., 2012; Viotti et al., 2013; Ebine et al., 2014). Regulators controlling these two vacuolar trafficking routes are obscure.

We have demonstrated previously that a multispan tonoplast protein Arabidopsis (Arabidopsis thaliana) PROTEIN S-ACYL TRANSFERASE10 (PAT10) mediates the S-acylation-dependent tonoplast association of CBL2, CBL3, and CBL6 (Zhou et al., 2013; Zhang et al., 2015). CBLs are calcineurin B-like proteins critical for Ca2+ signaling during plant growth and responses to...
RESULTS

Targeting of PAT10 to the Tonoplast Is Independent of Rab5-Mediated Post-Golgi Trafficking Routes

To test whether the vacuolar trafficking of PAT10 was independent of the classic post-Golgi trafficking route, we applied both genetic and pharmacological approaches. We first treated PAT10g-GFP:pat10-1 plants with the lipophilic dye FM4-64. Because the ectopic expression of PAT10 resulted in its mistargeting to the Golgi (Batistic, 2012; Qi et al., 2013), likely due to saturation of the ER sorting machinery, we analyzed the localization of PAT10 using PAT10g-GFP:pat10-1, in which all mutant phenotypes were rescued and the protein was targeted exclusively to the tonoplast (Zhou et al., 2013). As reported (Bolte et al., 2004), FM4-64 first labels the PM (Fig. 1A) and then internalizes to reach the tonoplast through endocytic trafficking (Fig. 1C). Treatment of the fungal toxin brevufungin A (BFA) resulted in the aggregation of internalized FM4-64 signals into so-called BFA compartments (Fig. 1B). However, the tonoplast association of PAT10 was not interfered with by BFA treatment (Fig. 1C), suggesting that the tonoplast targeting of PAT10 was independent of BFA-sensitive post-Golgi trafficking.

Because the classic post-Golgi trafficking routes require the activity of Rab5 (Uemura and Ueda, 2014), we expressed a dominant negative (DN) Rab5 to inhibit Rab5-dependent post-Golgi trafficking (Sohn et al., 2003; Lee et al., 2004). We used the promoter of CARPRICE (Pro_CPC) that is specifically active in nontrichoblast cells (N cells; Wada et al., 2002), while GFP-labeled PAT10 is expressed in both N cells and H cells (trichoblast cells).

Thus, targeting of the same transgene can be analyzed simultaneously in the same root under various conditions. The expression of Rab5DN also did not alter the tonoplast association of PAT10 (Fig. 1D). To verify that the genetic interference worked as it should, we examined the effect of Rab5DN on inositol transporter1 (INT1), whose tonoplast localization relies on Rab5-dependent vacuolar trafficking (Wolfenstetter et al., 2012). In contrast to the tonoplast association of INT1 in the wild type, both the PM and the tonoplast were labeled with INT1 signals in cells expressing Rab5DN (Supplemental Fig. S1). These results indicated that PAT10 did not take the classic Rab5-dependent vacuolar trafficking routes.

Fluorescence-Based Forward Genetic Screening to Identify Key Components Regulating Rab5-Independent Vacuolar Trafficking Routes

To determine the trafficking route of PAT10 to vacuoles and identify novel regulatory components in Rab5-independent trafficking routes, we performed fluorescence-based forward genetic screening. Because PAT10 is expressed in pollen and pollen tubes (Zhou et al., 2013), an in vitro pollen germination assay at the M1 generation would be able to detect mutations that resulted in the mistargeting of PAT10. We screened around 3,000 M1 plants from an ethyl methanesulfonate (EMS)-mutagenized PAT10g-GFP:pat10-1 population using fluorescence microscopy for pollen tubes that display aberrant targeting of PAT10-GFP. Two lines showed similar alterations at the subcellular distribution of PAT10-GFP (Supplemental Fig. S2). These two mutants are recessive, because PAT10-GFP was distributed to punctate vesicles in M2 roots but not in M1 roots. By crosses, we also verified that the two mutants were allelic, and thus named them reva1-1 and reva1-2 (for regulators of vacuolar trafficking1). Instead of the tonoplast distribution, PAT10-GFP was distributed as punctate vesicles (Fig. 2, A, C, E, and F). BFA treatment resulted in the accumulation of most PAT10-positive signals into a circle surrounding the FM4-64-labeled BFA cores (Fig. 2, B and D), indicative of its Golgi identity (Lam et al., 2009).

The Tonoplast Targeting of PAT10 Is Mediated by AP-3

To identify the casual gene for the reva1 mutants, we combined map-based cloning and sequencing (Supplemental Fig. S2). Rough mapping using F2 progeny derived from an outcross with Landsberg erecta (Ler) indicated that mutations were on chromosome 1. By sequencing, we identified a base pair change in the coding sequence of At1g48760, resulting in an amino acid substitution (G94D for reva1-1 and E278K for reva1-2; Supplemental Fig. S3). This gene encodes the ε-subunit of AP-3 (AP-3ε) (AP-3ε). Because two mutants of AP-3ε, the EMS-mutagenized pat4-1 and the T-DNA insertional mutant pat4-2, were identified previously (Feraru et al., 2010; Zwiewka et al., 2011), we renamed reva1-1 and reva1-2 as pat4-3 and pat4-4, respectively.
To verify that the tonoplast association of PAT10 depended on AP-3, we first analyzed its subcellular targeting in pat4-2, the null mutant allele of AP-3 (Feraru et al., 2010). As in pat4-3 and pat4-4 (Fig. 2), PAT10-GFP was distributed in punctate vesicles (Fig. 3A) that formed a ring-shaped structure surrounding the FM4-64 core upon BFA treatment in pat4-2 (Fig. 3B). By contrast, INT1, whose tonoplast association is independent of AP-3 (Wolfenstetter et al., 2012), was distributed at a ring-shaped membrane (Fig. 3C), as typical of vacuolar morphology in AP-3 mutants (Feraru et al., 2010; Zwiewka et al., 2011). To provide further evidence, we introduced ProUBQ10::RFP-SUC4 into PAT10g-GFP; pat4-2. As an AP-3-mediated tonoplast cargo, SUC4 was relocalized into cis-Golgi stacks in AP-3 mutants (Wolfenstetter et al., 2012). Indeed, PAT10-GFP and RFP-SUC4 overlapped substantially at punctate vesicles in pat4-2 (Fig. 3D), supporting PAT10 as the other AP-3 cargo. We also introduced fluorescent markers for the Golgi apparatus (WAVE22R; Geldner et al., 2009), for the TGN/EE (VHA-a1-RFP; Dettmer et al., 2006), and for the PVC/MVB (RFP-VSR2; Wang et al., 2011). As expected, PAT10 showed the most substantial colocalization with the Golgi marker (Fig. 3E) but not that of TGN/EE (Fig. 3F) or PVC/MVB (Fig. 1G). These results
demonstrated that the tonoplast association of PAT10 relies on AP-3 and that mutations at AP-3 resulted in the retention of PAT10 at the Golgi. To determine whether the tonoplast association of PAT10 was compromised in the mutants of other AP-3 subunits, we introduced PAT10-GFP into pat2-2 (a null mutant of AP-3b; Zwiewka et al., 2011), ap-3m-2 (a mutant of AP-3m; Kansup et al., 2013), and ap-3s (a mutant of AP-3s; Supplemental Fig. S4). Not surprisingly, all AP-3 mutants tested showed defective tonoplast targeting of PAT10-GFP (Supplemental Fig. S4). In addition, the expression of AP-3d-RFP driven by ProUBQ10 fully restored the tonoplast targeting of PAT10-GFP in pat4-2 (Supplemental Fig. S4), confirming the AP-3-dependent trafficking of PAT10.

Gly-94 and Glu-278 of AP-3d Are Key Residues Affecting AP-3 Complex Formation and Subcellular Targeting

All F1 plants from crosses between pat4-2 and pat4-3 or pat4-4 showed the same PAT10-GFP distribution pattern as the parental homozygous plants (Fig. 4, D-G), suggesting that both pat4-3 and pat4-4 are null mutants of AP-3d. Indeed, the Gly-94 and Glu-278 residues are evolutionarily conserved (Supplemental Fig. S3). To determine how these amino acid substitutions affected the function of AP-3, we first performed yeast two-hybrid (Y2H) assays between different AP-3 subunits. Coimmunoprecipitation showed that AP-3d may interact directly with AP-3m (Zwiewka et al., 2011). Using Y2H assays, we showed that the wild-type AP-3d but not the two mutated versions interacted with AP-3m (Fig. 4, A and B). Next, we expressed RFP translational fusions of AP-3d and the two mutant variants in root epidermal cells to determine their subcellular targeting. The wild-type AP-3d-RFP was localized at punctate vesicles, which accumulate into ring-shaped structure with faint signals also in the BFA core upon BFA treatment (Fig. 4, C and D), indicative of Golgi and TGN/EE identity. By contrast, the two AP-3d mutants showed diffused cytoplasmic localization (Fig. 4C). To provide further evidence of the subcellular targeting of...
AP-3, we generated transgenic plants expressing both AP-3 d-GFP and RFP-fused organelle markers, including WAVE22R for the Golgi (Geldner et al., 2009) and VHA-a1-RFP for the TGN/EE (Dettmer et al., 2006). Colocalization with these fluorescence markers showed that AP-3 associates with the Golgi and partially with the TGN/EE (Fig. 4, E and F). These results demonstrated that the two residues, Gly-94 and Glu-278, are critical not only for the formation of AP-3 but also its subcellular targeting.

CBL2 Targets to Both the Tonoplast and the PM in \textit{pat4-2} Depending on S-Acylation

\textit{PAT10} loss of function resulted in pleiotropic growth defects (Zhou et al., 2013), which is inconsistent with the fact that functional loss of AP-3 subunits only showed subtle defects in plant growth (Niihama et al., 2009; Feraru et al., 2010; Zwiewka et al., 2011), considering that \textit{PAT10} is mistargeted in the \textit{ap-3} mutants. To determine the reason behind the discrepancy, we analyzed the localization of CBL2 in \textit{ap-3} mutants, because the function of \textit{PAT10} was mediated mainly through the tonoplast association of CBL2 and CBL3 (Tang et al., 2012; Zhou et al., 2013). Interestingly, CBL2 was detected both at the PM and at the tonoplast in \textit{pat4-2} (Fig. 5B) and \textit{pat2-2} (Supplemental Fig. S5), in contrast to its tonoplast localization in the wild type (Fig. 5A). BFA treatment in \textit{pat4-2} and \textit{pat2-2} resulted in the accumulation of most CBL2 signals in the BFA compartment (Fig. 5C), overlapping with those of FM4-64 (Fig. 5C), indicating mis-localization of CBL2 in the mutants. Upon BFA treatment, no PM-associated signals were detected for CBL2 (Fig. 5C). Treatment of the protein S-acylation inhibitor 2-bromopalmitate (2-BP; Hemsley and Grierson, 2008; Zhou et al., 2013; Zhang et al., 2015) in the wild type resulted in the redistribution of CBL2 from the tonoplast into the cytoplasm (Fig. 5E), in contrast to treatment with dimethyl sulfoxide (DMSO; Fig. 5D). The same 2-BP treatment did not disturb the tonoplast association of INT1 (Fig. 5, H and I). In \textit{pat4-2}, 2-BP caused the redistribution of CBL2 through the tonoplast.
from both the PM and the tonoplast to the cytoplasm (Fig. 5, F and G), indicating that both the PM-associated and the tonoplast-associated CBL2 were modified by S-acylation. These results suggested that, in pat4-2 and in pat2-2, where PAT10 was retained at the Golgi, CBL2 was nonselectively transported through vesicular trafficking to the PM and to the vacuoles after being S-acylated at the Golgi.

AP-3-Dependent Vacuolar Trafficking Involves a Subpopulation of COPII

The tonoplast association of CBLs was reported to be independent of COPII based on genetic interference of Sar-1 or Sac12 (Batistic et al., 2010; Bottanelli et al., 2011). Sar-1 is a COPII-recruiting guanosine triphosphate-hydrolyzing protein (GTPase; Takeuchi et al., 2000) found at the ER exit site (ERES) but also over the ER network and mediating vesicle trafficking from the ER to the Golgi (Bassham et al., 2008; Chung et al., 2016).

To determine whether the tonoplast targeting of PAT10 relies on COPII, we expressed a DN-Sar-1b specifically in N cells using ProCPC. We used the ratio of tonoplast-associated fluorescence intensities between N cells and H cells to quantify the difference, which not only excludes the influence of different vacuolar morphology between N cells and H cells but also excludes the influence of differential transgene expression among different roots. By measuring the fluorescence intensities of PAT10-GFP on the tonoplast in N cells and H cells either from wild-type or DN-Sar-1b plants, we found that DN-Sar-1b did not interfere with the tonoplast targeting of PAT10 (Fig. 6, A, B, and D; Supplemental Data Set S1). However, the expression of DN-Sar-1b did compromise the tonoplast targeting of INT1 (Fig. 6, E, F, and H; Supplemental Data Set S1), such that GFP-INT1 showed a significantly reduced association at the tonoplast in DN-Sar-1b-expressing cells (Fig. 6, F and H; Supplemental Data Set S1) than in those of the wild type (Fig. 6, A and H; Supplemental Data Set S1).

A recent study indicated that, although different Sar-1s in Arabidopsis share high sequence homology, their function might be distinct (Zeng et al., 2015). Real-time quantitative PCR showed that Sar-1b and Sar-1c are constitutively expressed, while the transcript level of Sar-1a is low in all tissues examined (Supplemental Fig. S6). We also confirmed that Sar-1c is expressed in root
epidermal cells, where PAT10-GFP localization was analyzed (Supplemental Fig. S6). Thus, it was likely that only a subpopulation of COPII was involved in the transport of PAT10. To test this hypothesis, we generated ProCPC::DN-Sar-1c and analyzed its effect on the targeting of PAT10. Indeed, the expression of DN-Sar-1c substantially reduced the tonoplast association of both PAT10 (Fig. 6, C and D; Supplemental Data Set S1) and INT1 (Fig. 6, G and H; Supplemental Data Set S1) compared with the wild type (Fig. 6, A and G; Supplemental Data Set S1). These results suggested that the AP-3-mediated vacuolar trafficking of PAT10 was selectively transported at the ER by a subpopulation of COPII in which Sar-1c is a key component.

The HOPS Complex Is Required for AP-3-Mediated Vacuolar Trafficking

In yeast, the HOPS complex controls membrane fusion at the vacuoles (Nickerson et al., 2009). Interestingly, mutations either at AP-3 subunits or VPS41, a key component of HOPS, were able to suppress the zigzag growth of vti11 (Niihama et al., 2009). We thus considered the possibility of whether the function of HOPS was required for AP-3-mediated tonoplast targeting. It was reported recently that the functional loss of VPS41 resulted in male gametophytic lethality by interfering with in vivo pollen tube growth (Hao et al., 2016). Therefore, we decided to use the in vitro pollen tube growth system to examine the effect of VPS41 functional loss on the tonoplast targeting of AP-3 cargos. To this purpose, we introduced PAT10g-GFP into vps41-1/+, a null mutant for VPS41 (Hao et al., 2016). In wild-type pollen tubes, PAT10 and CBL2 were colocalized to filamentous and tubule structures, excluding from the apical region (Fig. 7A), which is typical for tonoplast distribution (Hicks et al., 2004). By contrast, PAT10 was distributed into small cytoplasmic vesicles in vps41-1 pollen tubes (Fig. 7A). In comparison, CBL2 was detected mostly at the PM, and to a lesser extent, cytoplasmic vesicles in vps41-1 (Fig. 7A). To exclude the possibility that the PAT10-positive vesicles were deformed vacuoles due to VPS41 loss of function, we stained pollen tubes with Oregon Green (OG). OG labels the dynamic tubular vacuoles of wild-type pollen tubes (Fig. 7, D and E), colocalizing with the tonoplast fluorescent marker WAVE9R (Geldner et al., 2009). By contrast, vacuoles of vps41-1 pollen tubes, as indicated by OG labeling, showed numerous granules (Fig. 7F), larger than the punctate vesicles where PAT10 was targeted to vps41-1 pollen tubes (Fig. 7A). These results indicated that the tonoplast association of PAT10 requires functional VPS41.

To determine whether it was the case for other AP-3 cargos, we also introduced ProUBQ10::GFP-VAMP711 in vps41-1. VAMP711 is a close homolog of the known AP-3 cargo VAMP713 (Ebine et al., 2011). We demonstrated that VAMP711 also relies on AP-3 for its tonoplast targeting by introducing ProUBQ10::GFP-VAMP711 in...
The tonoplast association of VAMP711 was compromised in \textit{pat4-2} such that signals were detected at the PM, cytoplasmic vesicles, and faintly at the tonoplast (Supplemental Fig. S7). Treatment of BFA resulted in the disappearance of GFP signals from the PM into BFA compartments, although a few deformed vacuoles in \textit{pat4-2} were still labeled with GFP-VAMP711 signals (Supplemental Fig. S7). These results suggested that VAMP711 relies mainly on AP-3 for its tonoplast targeting. Indeed, VAMP711 was relocated to cytoplasmic vesicles as well as the apical PM in \textit{vps41-1}, instead of the tonoplast (Fig. 7B). Interestingly, INT1, the cargo of the Rab5-mediated vacuolar trafficking route, also was mistargeted in \textit{vps41-1} pollen tubes (Fig. 7C), suggesting that the VPS41-participating HOPS complex is crucial for the vacuolar consumption of both AP-3-mediated and Rab5-mediated vesicles.

**DISCUSSION**

The Vacuolar Targeting of PAT10 Depends on AP-3

Several lines of evidence indicate that the multispan transmembrane protein PAT10 is transported to the tonoplast from the ER through AP-3-mediated trafficking (Fig. 8). First, the genetic interference of Rab5 did not affect the tonoplast association of PAT10 (Fig. 1), excluding the involvement of Rab5-mediated vacuolar trafficking. Second, interfering with the formation of Sar-1c-positive COPII by expressing DN-Sar-1c caused the reduced signal of PAT10 at the tonoplast (Fig. 6), indicating the passage of PAT10 through the Golgi during its transport. Third, PAT10 was retained at the Golgi in all AP-3 mutants tested (Figs. 2 and 3), similar to SUC4, another multispan transmembrane protein identified as an AP-3 cargo (Wolfenstetter et al., 2012).
Interestingly, unlike SUC4 and PAT10, the tail-anchored tonoplast protein VAMP711, a close homolog of the AP-3 cargo VAMP713 (Ebine et al., 2011), was not retained at the Golgi in AP-3 mutants (Supplemental Fig. S7). Instead, it was mistargeted to the PM in addition to the tonoplast (Supplemental Fig. S7), suggesting that different AP-3 cargos, once reaching the Golgi and in the absence of AP-3, are either retained at the Golgi or transported nonselectively through vesicular trafficking to the PM and the tonoplast.

Figure 7. The HOPS complex is required for AP-3-mediated vacuolar trafficking. A, CLSM of a wild-type (WT) or vps41-1 pollen tube coexpressing PAT10g-GFP and CBL2-RFP. Merges of the GFP and RFP channels are shown at the bottom. B and C, CLSM of a wild-type or vps41-1 pollen tube expressing GFP-VAMP711 (B) or GFP-INT1 (C). Merges of the GFP and transmission channels are shown at the bottom. Dotted lines illustrate the silhouettes of pollen tubes. D, CLSM of a wild-type pollen tube expressing WAVE9R (red) and stained with OG (green). E and F, CLSM of a wild-type (E) or vps41-1 pollen tube (F) stained with OG. Three-dimensional surface rendering images showing vacuolar structures are at the bottom. Bars = 10 μm.

Figure 8. Cartoon model showing the AP-3-dependent PAT10 trafficking. A, In the wild type (WT), PAT10 was selectively packed into the Sar1c-dependent COPII complex at the ER and destined for the Golgi. AP-3 mediates the transport of PAT10 from the Golgi to the vacuoles together with the HOPS complex, where cytosolic CBL2 is S-acylated and anchored to the tonoplast. B, In the mutants of AP-3, PAT10 was retained at the Golgi, where cytosolic CBL2 is S-acylated and anchored to the Golgi. Due to active vesicular trafficking at the Golgi, Sacylated CBL2 follows Rab5-dependent trafficking from the Golgi to the TGN/EE, where it targets to the PM or the tonoplast nonselectively.
PAT10-Mediated Membrane Anchorage of CBL2 Is Independent of Membrane Context

We at first excluded the possibility that AP-3 was involved in the tonoplast targeting of PAT10 for the simple reason that mutations in PAT10 resulted in strong developmental defects (Zhou et al., 2013) while ap-3 mutants were only mildly affected (Niihama et al., 2009; Feraru et al., 2010; Zwiewka et al., 2011). However, to our surprise, although PAT10 was retained at the Golgi (Figs. 2 and 3), its substrate CBL2 was targeted to the tonoplast and the PM in an S-acylation-dependent way (Fig. 5). The partial distribution of CBL2 at the tonoplast may be the reason that PAT10 was retained at the Golgi (Figs. 2 and 3), its substrate only mildly affected (Niihama et al., 2009; Feraru et al., 2010; Zhou et al., 2013). In ap-3 mutants, PAT10 was retained mostly at the Golgi (Figs. 2 and 3). Thus, S-acylation of CBL2 must have happened at the Golgi by the function of Golgi-retained PAT10. This would indicate that S-acylation by PAT10 is independent of its membrane context (i.e. either at the Golgi or the tonoplast) and that PAT10 is able to mediate the membrane anchorage of cytoplasmic CBL2. Because the Golgi is an endomembrane compartment constantly undergoing vesicle trafficking, the membrane-anchored CBL2 presumably followed vesicle flow nonselectively to the PM or the tonoplast through the TGN/EE (Figs. 5 and 7). Consistently, the tail-anchored tonoplast protein, VAMP711, also was nonselectively transported to the PM and the tonoplast through the TGN/EE in the absence of AP-3 (Supplemental Fig. S7). Indeed, ours (Supplemental Fig. S1) and previous results (Ebine et al., 2014) showed that inhibiting the Rab5-mediated vacuolar trafficking route resulted in mistargeting of tonoplast proteins to the PM, supporting the nonselective transport of Golgi-retained proteins in the case of disrupted vacuolar trafficking.

AP-3-Specific Vacuolar Trafficking of PAT10 Relies on the Sar-1c-Positive COPII Complex

COPII is an evolutionarily conserved complex mediating anterograde vesicular trafficking at the ER (Chung et al., 2016). It was proposed recently that different Sar-1 paralogs encoded in plant genomes may have distinct functions (Chung et al., 2016). Indeed, an amino acid difference determines the separate function of two Sar-1s (Zeng et al., 2015). The tonoplast association of CBL6 was unaffected by interfering with the formation of Sar-1b-positive COPII (Batistic et al., 2010), which is also true for PAT10 (Fig. 6). However, the fact that PAT10 is transported via AP-3 indicates its passage through the Golgi. Consistently, we have demonstrated that manipulating the activity of Sar-1c but not Sar-1b substantially compromised the tonoplast targeting of PAT10 (Fig. 6). How the specificity between different Sar-1s and cargos is determined requires further investigation.

The Tethering Complex HOPS Is Essential for AP-3- and Rab5-Mediated Vacuolar Trafficking

Compared with extensive studies on the trafficking routes vacuolar proteins take, little is known of the mechanism regulating the final arrival of vesicles at the tonoplast. We show here that both PAT10 and INT1, cargos mediated by AP-3 and Rab5, respectively, were mislocalized in vps41 (Fig. 7). In addition, functional loss of AP-3 resulted in the mistargeting of CBL2 to the PM and the tonoplast (Fig. 5), while CBL2 was detected only at the PM in vps41 pollen tubes (Fig. 7). These results indicate that VPS41 is critical for the vacuolar targeting of both AP-3- and Rab5-mediated vesicles. The essential requirement of HOPS to vacuolar fusion is consistent with the fact that functional loss of VPS41, and by inference the HOPS complex, resulted in male gametophytic lethality (Hao et al., 2016).

MATERIALS AND METHODS

Plant Materials and Growth Condition

Arabidopsis (Arabidopsis thaliana) plants were grown and transformed as described (Zhou et al., 2013). The Columbia-0 ecotype was used as the wild type. Transgenic plants were selected on one-half-strength Murashige and Skoog (MS) medium supplemented with 30 μg ml⁻¹ Basta salts (Sigma-Aldrich) or 50 μg ml⁻¹ hygromycin (Roche). The T-DNA insertion lines, SALK_076372 (vps41-1; Hao et al., 2016), SAIL_1258_G03 (pat2-2; Feraru et al., 2010), SALK_008818C (pat4-2; Zwiewka et al., 2011), SALK_064486 (ap-3-2; Kansup et al., 2013), and SAIL_269_F04 (ap-3r), were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). The T-DNA insertion line SALK_024964 (pat10-1) was described (Zhou et al., 2013). The pat4-3/vat1-1 and pat4-4/vat1-2 mutant alleles were isolated from an M2 population of PAT10-GFP/pat10-1 seeds mutagenized with EMS, which resulted in a nucleotide substitution at AP-38 (i.e. G281A or G323A, respectively). Fluorescence marker lines for coelating experiments, including WAVE2R (Geldner et al., 2009), WAVE9R (Geldner et al., 2009), and VHA-a1-RFP (Dettmer et al., 2006), were described previously.

DNA Manipulation

All constructs were generated using Gateway technology (Invitrogen) except where noted. Entry vectors for genes or fragments of interest were generated with the following primer pairs: ZP929/ZP930 for Sar-1b, ZP4302/ZP4303 for Sar-1c, ZP5324/ZP5325 for ProSAR1C, ZP1845/ZP1846 for INT1, ZP3601/ZP3602 for VAMP711, ZP4679/ZP4640 for SUC4, ZP4427/ZP4428 for AP-38 (without stop codon), ZP4427/ZP4406 for AP-36 (with stop codon), and ZP4210/ZP4208 for AP-3s. Dominant negative mutants of Rab GTPases were generated using the Phusion site-directed mutagenesis kit according to the manufacturer’s instructions from corresponding entry vectors with the following primer pairs: ZP1280/ZP1281 for Sar-1b-H74L, ZP4304/ZP4305 for Sar-1c-H74L, and ZP1275/ZP1276 for ARRA7-S24N (Rab5DN). All entry clones were generated in the pENTR/D/TOPO vector (Invitrogen). All PCR amplifications used Phusion hot-start high-fidelity DNA polymerase with the annealing temperature and extension times recommended by the manufacturer. All entry vectors were sequenced, and sequences were analyzed using Vector NTI (Invitrogen). The Bioneer PCR purification kit and Spin mini prep kit were used for PCR product recovery and plasmid DNA extraction, respectively.

Destination vectors for protein expression in planta, ProUBQ10-GW-GFP and ProUBQ10-GFP-GW, were modified by replacing the ProUBQ10 from ProSUC4-GFP-GW and ProSUC4-GFP-GW (Karimi et al., 2002), respectively, with ProUBQ10 (amplified with the primer pair ZP5301/ZP5311) using HindIII/Spel double digestion. A 1,252-bp sequence upstream of the CAPRICE start codon was amplified with ZP1257/ZP1258 and digested with SacI/Spel. The resulting fragment replaced the ProUBQ10 in ProUBQ10-RFP-GW and ProUBQ10-GFP-GW (Karimi et al., 2002) to generate the destination vectors ProUBQ10-RFP-GW and ProUBQ10-GFP-GW. The destination vector GW-GUS was described (Zhou et al., 2013). The destination vectors for RD and AD fusions in Y2H assays were pDEST32 and pDEST22, respectively (Invitrogen). Expression vectors were generated by LR reactions using LR Clonase II (Invitrogen). The expression vectors PAT10-GFP and ProUBQ10-CBL2-RFP were described previously (Zhou et al., 2013; Zhang et al., 2015). All primers are listed in Supplemental Table S1.
Genotyping PCR, Reverse Transcription-PCR, and Quantitative Real-Time PCR

Primers used to verify T-DNA insertions at the DNA level are as follows: ZP4408/ZP4449 for the wild-type copy and ZP4408/ZP1 for the mutant copy of VPS41 in SALK_076372 (vps1-1); ZP4702/ZP4703 for the wild-type copy and ZP4703/ZP4 for the mutant copy of AP-3β in SAIL_1258_C03 (pat2-2); ZP5639/ZP5640 for the wild-type copy and ZP5640/ZP1 for the mutant copy of AP-3β in SALK_069881C (pat4-2); ZP4394/ZP4705 for the wild-type copy and ZP4705/ZP1 for the mutant copy of AP-3α in SALK_064496 (ap-3α-2); and ZP4706/ZP4707 for the wild-type copy and ZP4707/ZP4 for the mutant copy of AP-3α in SAIL_269_F04 (ap-3α). Primers used to verify the mutant allele of ap-3 at the RNA level by reverse transcription-PCR are ZP4430/ZP4431. Primers used to perform quantitative real-time PCR are as follows: ZP5304/ZP5305 for ZP5304/ZP5305 for Sur-1a, ZP5306/ZP5307 for Sur-1b, and ZP5308/ZP5309 for Sur-1c. Total RNA extraction, reverse transcription-PCR, and quantitative real-time PCR were performed as described (Zhou et al., 2013). All primer sequences are listed in Supplemental Table S1.

EMS Mutagenesis and Map-Based Cloning

About 10,000 PAT10g-GFP;pat10-1 seeds were incubated with 25 mL of 0.2% (v/v) EMS in a 50-mL Falcon tube on a tube rotator for 15 h. The seeds were rinsed with water 10 times before being dried under a fume cupboard. In total, 3,000 M1 plants were analyzed by in vitro germination assay (Boavida and McCormick, 2007). Plants whose pollen tubes showed altered PAT10-GFP localization were selected for outcrosses. The mapping populations were generated by a cross between Ler and the corresponding mutant. We roughly mapped both pat4-3 and pat4-4 loci on chromosome 1 using an F2 mapping population (38 F2 progeny for pat4-3 and 30 F2 progeny for pat4-4). We used the simple sequence length polymorphism markers that can recognize polymorphisms between Columbia-0 and Ler based on information provided by TAIR.

Protein Interaction Assays

Y2H assays were performed using the Pro-Quest system (Invitrogen). YSD medium supplemented with all amino acids except Trp and Leu was used to select diploids. YSD medium supplemented with 15 mM 3-amino-1,2,4-triazole (5 mM 3-AT) at 30°C and all amino acids except Trp, Leu, and His was used to select colonies showing positive interactions. Quantification of β-galactosidase activity from Y2H experiments was performed as described previously (Huang et al., 2013).

Pharmacological Treatment

Stock solutions of various pharmacological drugs were prepared using DMSO as the solvent at the following concentrations: 20 mM 2-BP, 35 mM BFA, and 4 mM FM4-64. Stock solutions were diluted and added to one-half-strength MS medium at the following designated final concentrations: 20 mM 2-BP, 50 mM BFA, and 4 mM FM4-64. DMSO was equally diluted as the controls. All experiments were repeated at least three times. The 4-DAG seedlings were dipped in liquid one-half-strength MS medium supplemented with 4 μM FM4-64 (Invitrogen) for 5 min (except for the 1-min assay) at room temperature. Seedlings were then washed three times with liquid one-half-strength MS medium and treated with 50 μM BFA for 50 min prior to confocal fluorescence imaging. In the 2-BP treatment assay, 4-DAG seedlings were dipped in liquid one-half-strength MS medium supplemented with 20 μM 2-BP (Sigma) or DMSO overnight before imaging.

OG Staining of Growing Pollen Tubes

Fluorescence labeling of vacuoles in growing pollen tubes with the fluorescent cell-permeant dye OG 488 carboxylic acid diacetate (Invitrogen) was performed as follows: pollen grains were germinated on pollen germination medium (PCM) for 2.5 h, and then 100 μL of liquid PCM supplemented with 10 μM OG from a 10 mM stock solution in DMSO was dropped on in vitro growing pollen tubes for 50 min at 28°C in the dark. The pollen tubes were then washed with liquid PCM without OG before being imaged.

Fluorescence Microscopy

Fluorescence images were captured using a Zeiss LSM880 laser scanning microscope with a 40X/1.3 oil objective. GFP-RFP double-labeled plant materials were captured alternately using line switching with the multitrack function (488 nm for GFP and 561 nm for RFP). Fluorescence was detected using a 505- to 550-nm band-pass filter for GFP and a 575- to 650-nm band-pass filter for RFP. Image processing was performed with the Zeiss LSM image-processing software. The quantification of PAT10-GFP was presented using the tonoplast fluorescence intensity of N cells with Sar-1:DN expression versus those without. The average signal intensity of regions of interest at the tonoplast was measured using ImageJ.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: At5g55990 for CBL2; At2g46410 for CPC; At2g3330 for INT1; At1g51390 for PAT10; At4g19640 for RAB5; At1g09180 for Sur-1α; At1g56350 for Sur-1β; At4g02080 for Sur-1α; At4g23150 for VAMP711; At1g08190 for VPS41; At1g09960 for SLIC; At1g08570 for AP-3β; At3g55480 for AP-3β; At1g56590 for AP-3α; and At3g58080 for AP-3α.

Supplemental Data

The following supplemental materials are available.

- Supplemental Figure S1. The expression of Rab5DN interfered with the vacuolar targeting of INT1.
- Supplemental Figure S2. Identification of the rct1 mutants by EMS mutagenesis and cloning.
- Supplemental Figure S3. The Gly-94 and Glu-278 residues of AP-3α are evolutionarily conserved.
- Supplemental Figure S4. The tonoplast targeting of PAT10 is mediated by AP-3.
- Supplemental Figure S5. The tonoplast association of CBL2 depends on AP-3, but not Rab5-mediated post-Golgi trafficking.
- Supplemental Figure S6. Expression of Sar-1 homologs in Arabidopsis.
- Supplemental Figure S7. The tonoplast-targeted VAMP711 is another AP-3 cargo.
- Supplemental Table S1. Oligonucleotides used in this study.
- Supplemental Data Set S1. Source images and raw quantification data for Figure 6, D and G.

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LITERATURE CITED


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