Vacuoles are multifunctional organelles essential for the sessile lifestyle of plants. Despite their central functions in cell growth, storage, and detoxification, knowledge about mechanisms underlying their biogenesis and associated protein trafficking pathways remains limited. Here, we show that in meristematic cells of the *Arabidopsis thaliana* root, biogenesis of vacuoles as well as the trafficking of sterols and of two major tonoplast proteins, the vacuolar H\(^+\)-pyrophosphatase and the vacuolar H\(^+\)-adenosinetriphosphatase, occurs independently of endoplasmic reticulum (ER)–Golgi and post-Golgi trafficking. Instead, both pumps are found in provacuoles that structurally resemble autophagosomes but are not formed by the core autophagy machinery. Taken together, our results suggest that vacuole biogenesis and trafficking of tonoplast proteins and lipids can occur directly from the ER independent of Golgi function.

**INTRODUCTION**

The presence of a large central vacuole is one of the hallmarks of a prototypical plant cell. Vacuoles fulfill multiple functions that are essential for the lifestyle of plants. They are the main store for solutes and serve, through osmotic water uptake, as a hydrostatic skeleton that in combination with the cell wall provides the driving force underlying cell growth and reversible changes in cell volume. The success of land plants as sessile organisms is directly linked to the vacuole even under stressful conditions (Martinoia et al., 2007). Indeed, recent analysis of mutants has confirmed that the V-ATPase is of general importance for vacuolar transport (Krebs et al., 2010), whereas the role of the V-PPase, at least under nonstress conditions, seems to be limited to cytosolic PPi homeostasis (Ferjani et al., 2011). Both proton pumps are among the most abundant tonoplast proteins (Carter et al., 2004; Jaquinod et al., 2007; Schulze et al., 2012); however, the molecular mechanisms underlying their sorting and targeting remains to be identified. Assembly of the V\(_e\) subcomplex of the V-ATPase takes place in the endoplasmic reticulum (ER) and requires the presence of dedicated assembly factors (Neubert et al., 2008). The combined activity of the two enzymes enables plants to maintain transport into the vacuole even under stressful conditions (Maeshima, 2001; Gaxiola et al., 2007). V-ATPases are highly conserved, multisubunit proton pumps that consist of two subcomplexes, the peripheral V\(_{1}\) complex responsible for ATP hydrolysis and the membrane-integral V\(_{2}\) complex responsible for proton translocation (Matile, 1978; Marty, 1999; Jiang et al., 2000; Sze et al., 2002). In comparison, the V-PPase, a homodimer of a single polypeptide, is a much simpler enzyme that uses PPI to pump proton transport across the tonoplast (Maeshima, 2001; Gaxiola et al., 2007). Due to their different energy sources, it is generally assumed that the combined action of the two enzymes enables plants to maintain transport into the vacuole even under stressful conditions (Martinoia et al., 2007). Indeed, recent analysis of mutants has confirmed that the V-ATPase is of general importance for vacuolar transport (Krebs et al., 2010), whereas the role of the V-PPase, at least under nonstress conditions, seems to be limited to cytosolic PPI homeostasis (Ferjani et al., 2011). Both proton pumps are among the most abundant tonoplast proteins (Carter et al., 2004; Jaquinod et al., 2007; Schulze et al., 2012); however, the molecular mechanisms underlying their sorting and targeting remains to be identified. Assembly of the V\(_e\) subcomplex of the V-ATPase takes place in the endoplasmic reticulum (ER) and requires the presence of dedicated assembly factors (Neubert et al., 2008). We have shown previously that the subcellular distribution of the V-ATPase is determined by the isoform of the membrane-integral subunit VHA-a. Incorporation of VHA-a1 targets the complex to the trans–Golgi network (TGN)/early endosome (EE),
whereas complexes containing either VHA-a2 or VHA-a3 are found in the tonoplast (Dettmer et al., 2006; Krebs et al., 2010). The presence of the N-terminal cytosolic domain of VHA-a1 is sufficient for TGN/EE localization (Dettmer et al., 2006). Based on current knowledge of the trafficking routes to the tonoplast, this result would be most easily explained by the presence of a sorting motif that would cause retention of complexes containing VHA-a1, whereas VHA-a2 and VHA-a3 that lack such a motif would move on to their final destination, the tonoplast. However, the exact mode of targeting remains to be determined as it is becoming increasingly clear that proteins can reach the tonoplast using different routes that diverge after COPII-mediated ER export and differ in their requirement for the adaptor complex AP3 or their drug sensitivity (Pedrazzini et al., 2013). Importantly, it has to be considered that most studies addressing vacuolar targeting have been performed in tobacco (Nicotiana tabacum) leaf or suspension cells that contain a fully developed vacuole. By contrast, for meristematic cells in which vacuoles are still developing, tonoplast targeting is inseparably linked to the question of which compartment serves as the origin of the tonoplast membrane. Meristematic cells are generally assumed to contain multiple small, often tubular vacuoles that eventually fuse to give rise to the large central vacuoles found in elongating and mature cells (Taiz, 1992; Seguí-Simarro and Staehelin, 2006). The origin of lytic organelles in diverse eukaryotic cells is thought to be the TGN. This holds true for the central vacuole of plant cells (Marty, 1999), as well as fungal vacuoles (Bryant and Stevens, 1998) and animal lysosomes (Saffig and Klumperman, 2009). However, for plant vacuoles, evidence is limited to electron microscopy (EM) studies in which the presence of acid phosphatases and esterases in both the TGN and in young vacuoles was interpreted as evidence for vacuoles being derived from the TGN (Marty, 1978). In comparison, EM-based studies suggesting smooth ER tubules as the origin of the vacuole have received less attention (Matile and Staehelin, 2011). The presence of acid phosphatases and esterases in the central vacuolme and TGN compartments was interpreted as evidence for the TGN being the origin of the vacuole (Bryant and Stevens, 1998). This is surprising as the absence of PSVs in the root tip allows imaging of LV biogenesis along the developmental gradient from the quiescent center into the differentiation zone.

As a third alternative, it has been shown that in cells containing PSVs, LVs arise via cell type–specific transformation pathways (Zheng and Staehelin, 2011). Several Arabidopsis thaliana mutants lacking vacuoles or with altered vacuole morphology have been characterized (Rojo et al., 2001; Feraru et al., 2010; Isono et al., 2010), but vacuole biogenesis in the Arabidopsis root has not been analyzed systematically. This is surprising as the absence of PSVs in the root tip allows imaging of LV biogenesis along the developmental gradient from the quiescent center into the differentiation zone.

Here, we thus employed multiple tools for genetic and pharmacological interference in combination with live-cell imaging, three-dimensional (3D) reconstruction, and EM. We show that in meristematic cells of the Arabidopsis root, vacuole biogenesis and trafficking to the tonoplast of the two proton pumps V-ATPase and V-PPase occur independently of ER-Golgi and post-Golgi trafficking. Instead, both pumps are found in provacuoles that structurally resemble autophagosomes but are formed without the core autophagy machinery. Taken together, our results suggest that vacuole biogenesis and trafficking of tonoplast proteins and lipids can occur directly from a subdomain of the ER independent of Golgi function.

RESULTS

 Trafficking to the Tonoplast Is Independent of Golgi Function and COPII-Mediated ER Export

The localization of the Arabidopsis V-ATPase is determined by the isoform of subunit VHA-a that is assembled into the multisubunit enzyme complex. Whereas Vacuolar H+-ATPase subunit a1 (VHA-a1) targets the V-ATPase to the TGN/EE, complexes containing VHA-a2 and VHA-a3 are localized to the tonoplast (Dettmer et al., 2006). Assembly of the V-ATPase occurs in the ER (Herman et al., 1994) and requires the action of ER-resident assembly factors (Graham et al., 2003; Neubert et al., 2008). However, in meristematic root cortex cells of seedlings expressing VHA-a3-GFP (for green fluorescent protein) and the ER intrinsic membrane protein and putative V-ATPase assembly factor VMA12 fused to red fluorescent protein (RFP), the two fluorescent signals were completely separate (Figure 1A). Taking the maturation time of GFP (Iizuka et al., 2011) into account, we used the faster maturing VHA-a3-mRFP (Campbell et al., 2002; Brüx et al., 2008) in pharmacological and genetic approaches to determine the route of VHA-a3-containing complexes from the ER to the tonoplast in root meristematic cells (Figure 1B). In agreement with our previous finding that the V-ATPase inhibitor concanamycin A (ConcA) blocks post-Golgi trafficking of secretory and endocytic cargo (Viotti et al., 2010; Scheuring et al., 2011), the tonoplast myo-inositol transporter INT1 (Wolfenstetter et al., 2012) fused to GFP (INT1-GFP) was trapped in intermediate compartments labeled by the endocytic tracer FM4-64 after ConcA treatment (see Supplemental Figure 1A online). By contrast, ConcA did not cause accumulation of newly synthesized VHA-a3 in the TGN/EE-derived aggregates, as indicated by the lack of colocalization between VHA-a1-GFP and VHA-a3-mRFP (Figure 1C) or VHA-a3-GFP and FM4-64 (see Supplemental Figure 1B online).

As INT1 has been shown to be delivered to the vacuole in an adaptor protein complex 3 (AP3)–independent manner (Wolfenstetter et al., 2012), we next analyzed if trafficking of VHA-a3 follows the AP3-dependent post-Golgi transport route to the vacuole. When VHA-a3-GFP was expressed in the pat2-1 mutant lacking the β-subunit of the AP3 complex (Feraru et al., 2010), it was clearly detectable at the tonoplast but not in endosomal compartments (see Supplemental Figure 1C online). As blocking post-Golgi transport did not affect VHA-a3 trafficking, we next investigated if VHA-a3 is transported from the ER to the Golgi apparatus. GNL1 is a guanine-nucleotide exchange factor (GEF) of the ADP-ribosylation factor (ARF) family of GTPases that mediates ER-Golgi transport, and it is resistant to the fungal toxin Brefeldin A (BFA) (Richter et al., 2007; Teh and Moore, 2007). By rendering GNL1 BFA sensitive (GNL1-BFA3), ER export of VHA-a1 can be blocked by BFA treatment (Richter et al., 2007). In order to test whether VHA-a3 behaves similarly, we coexpressed VHA-a1-GFP and VHA-a3-mRFP in gnl1;gnl1-BFA3 seedlings. Upon BFA treatment, VHA-a1-GFP but not VHA-a3-mRFP accumulated in the ER (Figure 1D). Using fluorescence recovery after photobleaching (FRAP) experiments (Figures 1E and 1F), we showed that VHA-a3-mRFP signal at the tonoplast recovered after 3 h without detectable accumulation in
the ER, whereas in the same cells, VHA-a1-GFP was retained in the ER. Treatment with cycloheximide (CHX) prevented FRAP, indicating that recovery is not based on diffusion from unbleached regions (Figures 1E and 1G).

Based on these observations, we hypothesized that VHA-a3 trafficking to the tonoplast is Golgi independent. To further investigate this at the ultrastructural level, we used immunocytochemistry on ultrathin sections as a more sensitive method to detect either VHA-a3-GFP (Figure 2A) or the V-PPase AVP1/VHP1 (Figure 2B). Whereas both proteins were clearly detectable at the tonoplast (Figures 2A to 2C), we obtained a much higher immunogold labeling density for AVP1/VHP1 and thus focused on the quantitative analysis of this antigen in the compartments of the endomembrane system. AVP1/VHP1 was neither detectable in the boundary membrane of the multivesicular bodies (MVBs) (Figures 2D and 2G) nor in the TGN/EE, Golgi stacks, or the ER (Figure 2G). ConcA treatment did also not lead to accumulation in the resulting Golgi/TGN hybrids (Figures 2E to 2G). We thus concluded that in meristematic cells of the root tip, both proton pumps are delivered to the tonoplast by a coat protein complex II (COPII)- and Golgi-independent trafficking pathway.

**Figure 1.** Golgi-Independent Transport of the V-ATPase and the V-PPase AVP1/VHP1 to the Tonoplast.

(A) to (G) CLSM of cortex cells in the wild type (A) and gni1;GNI1-BFA (B) to (G).

(A) In cells expressing VHA-a3-GFP and VMA12-RFP, VHA-a3-GFP cannot be detected in the ER.

(B) In untreated cells, VHA-a1-GFP labels the TGN/EE, whereas VHA-a3-mRFP is found at the tonoplast.

(C) After 3 h in the presence of ConcA, VHA-a1-GFP and VHA-a3-mRFP do not colocalize.

(D) After 3 h in the presence of BFA, VHA-a1-GFP is retained in the ER while VHA-a3-mRFP is not.

(E) to (G) FRAP experiment showing that in the presence of BFA, VHA-a3-mRFP is newly synthesized but does not accumulate in the ER. Bars = 10 μm.

(F) and (G) CLSM images showing the region of interest (dashed box) before, immediately after, and 200’ after photobleaching in the presence of BFA (F) or BFA + CHX (G).

**Trafficcking of Sterols to the Tonoplast**

VHA-a3 has been detected in detergent-resistant membranes (DRMs; Keinath et al., 2010), and we next employed the 3-β-hydroxysterol-specific, sterol binding probe filipin III (Boutté et al., 2010, 2011) to determine if VHA-a3 is present in sterol-enriched membranes. In order to test whether filipin-sterol complexes can be found at vacuolar membranes, we performed detection of filipin-sterol fluorescence on a VHA-a3-GFP transgenic line. Filipin-sterol and VHA-a3-GFP fluorescence indeed
partially colocalized in both meristematic (Figure 3A) and elongated (Figure 3B) root cells. Moreover, ultrastructural analysis revealed typical 20- to 30-nm deformations of filipin-sterol complexes at the tonoplast, confirming the presence of sterols in this membrane (Figure 3C). Sterols and the syntaxin KNOLLE have previously been demonstrated to substantially colabel TGN membranes in cytokinetic cells (Boutté et al., 2010), and we confirmed substantial colabeling in BFA-treated cells (Figure 3D). Intriguingly, however, when we blocked ER-Golgi transport in the gnl1 mutant or gnl1;GNL1-BFA by BFA treatment, membranes highlighted by filipin-sterol fluorescence could be efficiently distinguished from the ER (Figures 3E and 3F, top panels) in which KNOLLE is retained under these conditions. By contrast, clear colabeling between KNOLLE and sterols was observed in gnl1 and gnl1;GNL1-BFA lines not subjected to BFA treatment (Figures 3E and 3F, bottom panels). Hence, BFA interference with ER-Golgi trafficking efficiently separates the sterol and KNOLLE trafficking routes and sterols can still reach vacuolar membranes.

Vacuole Biogenesis in the Arabidopsis Root Tip

The results presented so far point to a Golgi-independent trafficking route to the tonoplast in root tip cells. To be able to define this route, we first needed to describe the LVs present in meristematic root cells at improved spatial and temporal resolution. An ultrastructural overview of the root meristem showed that vacuoles of variable size could be detected in all cells of the root meristem, including the quiescent center and the surrounding initials (Figure 4A). In single EM sections of meristematic cells, multiple small vacuoles were detected, some of which appeared tubular (Figure 4B). Vacuoles were also present during cytokinesis (Figure 4C). In roots stained with FM4-64 and the green fluorescent pH probe 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) that accumulates efficiently in the vacuolar lumen (Swanson and Jones, 1996; Krebs et al., 2010), vacuoles could be detected in all cell types, with the exception of stele and columella cells (Figure 4D). BCECF-based pH measurements revealed that vacuoles in the meristematic region of the root are indeed acidified (see Supplemental Figure 2 online). 3D projections of Z-stacks (Figures 4E and 4G) and surface renderings (Figures 4F and 4H) showed that each cell contains a single complex tubular network that is equally distributed among the two daughter cells during cytokinesis (Figures 4G and 4H; see Supplemental Movies 1 and 2 online). Determination of vacuolar surface area and volume based on 3D rendering showed that the surface/volume ratio declined from 3.5 for cells in the meristematic zone to 0.5 for cells in the differentiation zone (see Supplemental Figure 3 online).

As we could detect VHA-a3-GFP but not BCECF staining in the stele, we used the red fluorescent pH probe carboxy-SNARF to determine the structure of vacuoles in provascular cells. Compared with epidermis and cortex cells, provascular cells

![Figure 2](http://example.com/figure2.png)

Figure 2. Quantitative Analysis of AVP1/VHP1 Distribution in the Endomembrane System.

(A) to (C) Immunogold EM detects VHA-a3-GFP ([A], arrowheads) and AVP1/VHP1 ([B] and [C]) at the tonoplast.

(D) AVP1/VHP1 is detected at the tonoplast but not in the limiting membrane of MVBs (arrowheads).

(E) and (F) Upon 12 h treatment with ConcA, AVP1/VHP1 is not detected in Golgi stacks or TGN-derived elements despite an unchanged amount of signal at the tonoplast. G, Golgi; V, vacuole. Bars = 400 nm in (A) to (F).

(G) Quantification of labeling density (gold particles per micrometer of membrane) at vacuoles (Vac), provacuoles (PRV), MVB, TGN, Golgi, ER, and plastids/mitochondria (P/M). Error bars represent the so for n = 30.
displayed a much finer tubular network (Figures 5A to 5C). Interestingly, not all structures marked by VHA-a3-GFP were also stained by carboxy-SNARF (Figure 5C). Similarly, VHA-a3-mRFP loops with limited BCECF staining were frequently detected in meristematic cells but not in cells in the elongation zone (Figures 5D to 5G). The VHA-a3-mRFP fluorescence intensity in such loops was found to be twofold higher than on membranes enclosing BCECF (Figure 5H). Using time-lapse imaging, we were able to follow the conversion of a loop labeled by VHA-a3-mRFP to a tubular structure filled with BCECF (Figure 5I; see Supplemental Movie 3 online).

On the ultrastructural level, the VHA-a3-mRFP loops could correspond to circular structures consisting of two closely apposed membranes that seemingly engulf a portion of cytosol (Figure 6A). To determine if such double-membrane structures indeed represent an early step in vacuole biogenesis, we next used immunocytochemistry against AVP1/VHP1 or VHA-a3-GFP on high-pressure frozen and freeze-substituted samples. AVP1/VHP1 immunocytochemistry allowed tracking of the tonoplast in root cells and confirmed the presence of complex vacuolar networks (see Supplemental Figures 4A to 4C online). Focusing in particular on cells in the vicinity of the quiescent center, we noticed that anti-AVP1/VHP1 immunogold labeling indeed marked circular structures. Consecutive sections revealed a 30-nm-thick spherical double bilayer enclosing a portion of cytoplasm (Figure 6B). Further analysis showed that these either spherical or semispherical structures often showed an enlarged lumen in different locations (Figures 6C and 6D; see Supplemental Figure 4D online) or through the whole perimeter (see Supplemental Figures 4E and 4F online). The presence of AVP1/VHP1 and VHA-a3-GFP

**Figure 3. Sterol Enrichment at the Tonoplast Occurs Independently of Transport via the Golgi.**

(A) Filipin-sterol fluorescence colocalizes with VHA-a3-GFP (arrow) in young cells lacking a large central vacuole.

(B) Filipin-sterol signal colocalizes with VHAa3-GFP at the tonoplast in elongated cells.

(C) Ultrastructural analysis reveals 20- to 30-nm membrane deformations (arrowheads) typical of filipin-sterol complexes at the tonoplast of young and mature vacuoles (left) but absent from vacuoles of samples treated with DMSO solvent (right). V, vacuole.

(D) In Col-0 seedlings treated with BFA for 2 h, filipin-sterol fluorescence colabels with the cytokinesis-related syntaxin KNOLLE (KN) in BFA bodies, but also stains additional membranes that are neither part of the cell plate nor of BFA bodies (arrows).

(E) In gnl1 treated with BFA (top panels), while KN is retained in the ER, filipin-sterol fluorescence mostly highlights membranes distinct from the ER (arrows). In untreated gnl1 (bottom panels), colabeling between KN and sterols was observed.

(F) In gnl1/GNL1-BFAS treated with BFA (top panels), while KN is retained in the ER, filipin-sterol fluorescence mostly highlights membranes distinct from the ER. In untreated gnl1/GNL1-BFAS (bottom panels), colabeling between KN and sterols was observed. Bars = 10 μm in (A), (B), and (D) to (F) and 400 nm in (C).
the fusion event between these structures and MVBs (see Supplemental Figure 4G online) suggested that the structures indeed represent early stages of vacuole biogenesis and can therefore be referred to as provacuoles. Constrictions that seemingly separated two adjacent lumina represent an additional feature of provacuoles (Figure 6F).

Post-Golgi Trafficking and Autophagy Are Not Required for Provacuole Formation

To determine the origin of provacuoles, we next analyzed the effects of impaired post-Golgi trafficking. When VHA-a3-GFP was expressed in the AP3-deficient pat2-1 mutant, both number and fluorescence intensity of loops was found to be increased (Figures 7A to 7C), which is in agreement with the abnormal multilayered vacuolar enclosures reported in elongating cells of this mutant (Feraru et al., 2010).

Ultrastructural analysis confirmed that provacuoles in pat2-1 were often multilamellar (Figure 7D; see Supplemental Figure 5A online). Multilayered provacuoles were also found in a VACUOLAR PROTEIN SORTING45 (VPS45)–RNA interference line (Figure 7E). VPS45 is a positive regulator of the TGN-localized Syntaxin of plants41/Syntaxin of plants61/vesicle transport through interaction with t-SNAREs SNARE complex, which is involved in trafficking of vacuolar cargo (Bassham et al., 2000). For both pat2-1 and the VPS45-RNAi line, impaired post-Golgi trafficking affected the morphology of AVP1/VHP1-positive provacuolar...
Figure 5. Early Stages of Vacuole Formation.

(A) to (C) Developing vacuoles in stele cells visualized by combining VHA-a3-GFP (A) with luminal staining by SNARF-1 (B). Arrowheads point to structures positive for VHA-a3-GFP but lacking luminal staining (C).

(D) to (F) Developing vacuoles in cortex cells visualized by combining VHA-a3-mRFP (E) with luminal staining by BCECF (D). Arrowheads point to loop structures positive for VHA-a3-mRFP but lacking luminal staining (F).

(G) Quantification of VHA-a3-mRFP loops with low luminal BCECF staining in cells of the meristematic and elongation zones. Error bars indicate the SD for n = 10 roots.

(H) Fluorescence intensity profile of a loop and a vacuole (white line) indicating that VHA-a3-mRFP fluorescence in loops is twofold higher than in the neighboring tonoplast.

(I) Time-lapse imaging showing the conversion of a loop labeled by VHA-a3-mRFP to a tubular structure filled with BCECF (see Supplemental Movie 3 online).

Bars = 10 µm in (A) to (F) and (H) and 5 µm in (I).
membranes but did not prevent their formation. This observation was confirmed by blocking vacuolar transport pharmacologically using ConcA. Multilamellar provacuoles were detected after 1 h of exposure to ConcA (Figure 7F), and extended treatments up to 12 h led to enhancement of the effect (see Supplemental Figures 5B and 5C online). Moreover, long-term ConcA treatment blocked cell plate formation but not the biogenesis of vacuoles (see Supplemental Figure 4C online). Additional evidence that the donor membrane for provacuole formation cannot be the TGN was provided by the analysis of the gnl1 mutant upon extended BFA treatment. The complete abolition of Golgi and post-Golgi transport caused by the collapse of COPII-mediated export from the ER did not prevent the formation of provacuoles (Figure 7G).

The amsh3 mutant has been reported to have a strong defect in vacuole biogenesis (Isono et al., 2010). However, our ultrastructural analysis revealed that in amsh3 root cells, provacuoles were present but of either normal or aberrant morphology (Figure 7H). Interestingly, accumulation of autophagosomes has been reported in amsh3 mutant seedlings (Isono et al., 2010). Similarly, we observed a high number of structures reminiscent of autophagosomes in amsh3, but as it is known that autophagosomes become acidic only after fusion with either endosomes or lysosomes (Klionsky et al., 2008), the presence of AVP1/VHP1 on their membranes strongly suggested that they represented provacuoles. Indeed, double-membrane structures with and without AVP1/VHP1 labeling were observed in gnl1 seedlings after BFA treatment (Figure 7I). Given the structural resemblance of provacuoles and autophagosomes and the fact that autophagy has been proposed to be involved in vacuole biogenesis (Marty, 1999), we analyzed provacuole formation in atg2-1, atg5-1, and atg7-2 mutants that are unable to form autophagosomes (Thompson et al., 2005; Inoue et al., 2006; Hofius et al., 2009). Intriguingly, we observed that provacuoles were formed in all three atg mutants and that the morphology of their vacuoles was not altered (Figures 7A and 7J; see Supplemental Figures 5D and 5E online). Moreover, SNARF-1 staining of seedlings expressing the autophagy marker Atg8b confirmed that most autophagosomes are not acidic (Figure 7K) and that Atg8b-GFP does not accumulate at provacuolar loops (Figure 7L). Taken together, our data strongly suggests that provacuoles can be distinguished from autophagosomes and that the Atg machinery is not involved in vacuole biogenesis.

**Is the ER the Origin of Provacuoles?**

Since provacuole formation did not require Golgi function, post-Golgi trafficking, or the core autophagy pathway, we used live-cell imaging to investigate if provacuoles originate directly from the ER. However, even in a transgenic line coexpressing the V-ATPase assembly factor VMA21-GFP (Neubert et al., 2008) and VHA-a3-mRFP, the V-ATPase subunit was detectable only in small vacuoles nested within the network of the ER (Figures 8A and 8B). Although assembly of the V-ATPase takes place in the ER, we were not able to detect VHA-a3-mRFP in the ER.
Figure 7. Provacuoles Do Not Originate from Post-Golgi Trafficking and Are Distinct from Autophagosomes.

(A) to (C) Quantification of loops in the wild type and pat2-2 showing that pat2-2 has more loops with a higher fluorescence intensity in the meristematic zone (B) as well as in the elongation zone (C). Error bars for the number of loops in (A) represent the SD for n = 10 roots and for the fluorescence intensity of n = 65 loops.

(D) to (I) Immunogold cytochemistry of AVP1/VHP1 on ultrathin sections after high-pressure freezing and freeze substitution. (D) Ultrastructural analysis of the pat2-1 mutant. Provacuoles are present but display an aberrant multilayered morphology.
indicating that the assembled complexes are rapidly and efficiently moved away from the assembly site. Given the limited spatial resolution of these confocal laser scanning microscopy (CLSM) images, it could neither be excluded nor proven that at least some of the provacuoles are still physically connected to the ER. We thus used ultrastructural analysis, and in rare cases, we were indeed able to detect direct connections between ER and provacuoles at the ultrastructural level (Figure 8C). The presence of the ER chaperone calnexin on provacuoles as detected by immunogold labeling provides further supportive evidence (see Supplemental Figure 6 online).

Taken together, our data strongly suggest that provacuoles are formed from a subdomain of the ER in which proteins including V-ATPase and V-PPase are enriched by a yet to be identified mechanism that might involve changes in lipid composition (Figure 8D).

**DISCUSSION**

**Traffic Routes to the Tonoplast**

Although the V-ATPase and the V-PPase belong to the most abundant tonoplast proteins, their traffic routes have not been determined. Earlier studies have shown that both enzymes can also be detected in other compartments of the endomembrane system, including the ER, Golgi, and endosomes, and, at least in certain cell types or species, even at the plasma membrane (PM; Herman et al., 1994; Ratajczak et al., 1999; Dettmer et al., 2006; Paez-Valencia et al., 2011). Both enzymes are present in multiple isoforms (Sze et al., 2002; Segami et al., 2010), and their localization has mostly been determined using polyclonal antibodies that did not allow differentiation between individual isoforms (Seidel et al., 2008). We have shown previously that subcellular distribution of the Arabidopsis V-ATPase is determined by the presence of the respective isoforms of the membrane-integral subunit VHA-a (Dettmer et al., 2006), and here we tracked fully functional fluorescent fusion proteins of VHA-a3 on their way to the tonoplast of the LV in Arabidopsis root tip cells. Functional fluorescent fusion proteins for AVP1/VHP1, the only type I H+-PPase of Arabidopsis, have not been reported. However, a peptide antibody (Kobae et al., 2006) allowed us to specifically detect AVP1/VHP1 in immunocytochemistry on ultrathin sections, and the signal was exclusively detected at the tonoplast in Arabidopsis root tip cells. At least in this tissue, AVP1/VHP1 can thus be considered as a true vacuolar H+-PPase (V-PPase).

 Trafficking of membrane-integral proteins to the tonoplast starts with cotranslational insertion into the ER membrane and is followed by, once quality control has been passed, subsequent COPII-mediated transport to the Golgi. A recent study in Arabidopsis mesophyll protoplasts has shown that at the Golgi, proteins are sorted into either the AP3-dependent or AP-3 independent pathway (Wolfenstetter et al., 2012). We have shown previously that ConcA treatment blocks transport of soluble as well as endocytic cargo to the vacuole and integral membrane proteins that pass the TGN on their way to the tonoplast should thus also be trapped after ConcA treatment (Dettmer et al., 2006; Vioiti et al., 2010). This was indeed the case for the myo-inositol transporter INT1; however, ConcA did not cause accumulation of either VHA-a3 or AVP1/VHP1 in the resulting Golgi-TGN hybrid structures. As INT1 has been shown to follow the AP-3-independent pathway, it will be interesting to determine if other proteins taking this route will also be blocked by ConcA treatment. Blocking the AP-3-dependent pathway leads to accumulation of SUC4-GFP in the cis-Golgi (Wolfenstetter et al., 2012), and it seems possible that this pathway remains operational after ConcA treatment. Nevertheless, we have shown here that in the pat2-1 mutant, lacking the β-subunit of the AP3 complex (Feraru et al., 2010), VHA-a3 and AVP1/VHP1 still reach their destination pointing to the existence of a third route to the tonoplast of the LV that might completely bypass the Golgi apparatus. Although mammalian isoforms of subunit a have been shown to be modified by N-glycosylation (Kartner et al., 2013), VHA-a3 lacks predicted N-glycosylation sites and passage through the Golgi is thus not required.

In cell types in which the ARF-GEF proteins involved in ER-Golgi transport happen to be sensitive to BFA, the drug causes fusion of both compartments and can thus be used to block ER export (Nebenführ et al., 2002). GNL1, the respective ARF-GEF protein expressed in the root tip, is BFA resistant, and BFA can thus not be used to block ER export in wild-type roots. As it had been shown previously that in gnl1 plants expressing a BFA-sensitive variant of GNL1, ER export of complexes containing VHA-a1-GFP is efficiently blocked (Richter et al., 2007), we introduced VHA-a3-mRFP into this genetic background and could show that, unlike VHA-a1-GFP, it does not accumulate in the ER. Considering that in the same cells the less abundant VHA-a1 fused to the comparatively slow maturing GFP is clearly detectable in the ER, it seems unlikely that accumulation of VHA-a3-mRFP was below the detection limit. Furthermore, we performed FRAP experiments to provide evidence that BFA treatment does not cause an inhibition of VHA-a3-mRFP synthesis.

**Figure 7.** (continued).

(E) Ultrastructural analysis of a VPS45-silenced line (siVPS45). Meristematic root cells contain multilayered provacuoles.

(F) Treatment with ConcA induces formation of multilayered provacuoles after 1 h of treatment.

(G) Upon 6 h of BFA treatment of the gnl1 mutant, provacuoles were still present.

(H) In the amr3-1 mutant, morphologically aberrant provacuoles were present, sometimes with multilayered profiles.

(I) In the BFA-treated gnl1 mutants, double membrane structures without immunogold labeling are observed next to AVP1-labeled provacuoles.

(J) In the auz2-1 mutant defective in autophasosome formation, provacuoles of normal morphology are present.

(K) and (L) Confocal microscopy of seedlings expressing GFP-ATG6b stained with SNARF-1. Autophasosomes labeled with GFP-ATG6b are distinct from the acidified vacuolar lumen (K) and do not accumulate on provacuolar loops (L).

Rectangles indicate magnified areas in (E), (F), (J), and (I). Bars = 10 µm in (B), (C), and (K), 1 µm in (L), 400 nm in (D) to (H) and (J), and 200 nm in (I).

10 of 16 The Plant Cell
BFA-insensitive trafficking to the tonoplast has been reported for several integral membrane proteins but many of them were specifically targeted to PSVs (Gomez and Chrispeels, 1993; Pedrazzini et al., 2013). Interestingly, one of the main arguments in favor of the multivacuole hypothesis or, more specifically, the simultaneous existence of PSVs and LVs has been the identification of different sorting routes (Paris et al., 1996; Jiang and Rogers, 1998). PSVs are largely absent from root tips of Arabidopsis seedlings, and it will thus be of interest to extend our studies to tissues that contain PSVs. Most importantly, the mechanism underlying differential targeting of VHA-a1 and VHA-a3 complexes needs to be determined.

**Figure 8. Do Provacuoles Originate from the ER?**

(A) and (B) CLSM codetection of VHA-a3-mRFP and its assembly factor VMA21-GFP in root cortex cells reveals lack of colocalization in the ER and small vacuoles devoid of VMA21-GFP at the periphery of the nuclear envelope. (C) Provacuole connected to the cortical ER as observed by immunogold EM with anti-AVP1/VHP1 after ConcA treatment. (D) Schematic model of vacuolar biogenesis. V-ATPase assembly takes place in the ER (I). V-ATPase and V-PPase are sorted into a subdomain of the smooth ER that differs in lipid composition (II). The resulting double-membrane sheet curves into a cup-shaped structure with minimal luminal volume (III). Increase in luminal volume leads to formation of ball-shaped or tubular structures (IV). Fusion with preexisting vacuoles can occur at any stage (V). Bars = 10 µm in (A) and (B) and 400 nm in (C).
Direct Delivery from the ER to the Tonoplast

Incorporation of membrane-integral proteins into COPII vesicles requires interaction with Sec24 mediated by either diacidic, dihydrophobic, or diaromatic motifs (Hanton et al., 2006). Although such motifs are present in VHA-a1 and VHA-a3 as well as AVP1/VHP1, their functional relevance remains to be determined. In yeast, subunit a of the V-ATPase is the subunit present only in two isoforms, Stv1p and Vph1b, that are responsible for differential targeting of the V-ATPase to the Golgi-endosomal network or the vacuolar membrane. A tripeptide motif in the N terminus of Stv1p has recently been shown to be required for correct localization (Finnigan et al., 2012); however, this motif is not conserved in VHA-a1. VHA-a2 and VHA-a3 have been detected in DRMs (Keinath et al., 2010). Interestingly, the authors of this study have analyzed DRMs in PM-enriched samples and have thus interpreted the presence of V-ATPase subunits in their samples as evidence for the localization and potential function of the V-ATPase at the PM. We have so far been unable, by either CLSM or EM, to confirm PM localization of the Arabidopsis V-ATPase. Alternatively, the presence of VHA-a2 and VHA-a3 in DRMs could simply be due to residual tonoplast membranes in the PM-enriched samples. Indeed, the presence of DRMs in tonoplast preparations has recently been confirmed (Ozolina et al., 2012) and is in agreement with the fact that both sterols and sphingolipids, two classes of lipids typically enriched in DRMs, are present in amounts comparable to the PM (Yoshida and Uemura, 1986). Here, using the sterol binding probe filipin III, we have not only shown that sterols are indeed present in the tonoplast of Arabidopsis root tip cells but also that they still reach the tonoplast when ER export is blocked by BFA treatment in the sensitized GNL1-BFA<sup>A</sup> background. Although sterol biosynthesis takes place at the ER, sterols do not accumulate in this compartment (Boutté and Grebe, 2009). In fungal and mammalian cells, export of sterols is mediated by oxysterol binding proteins, but comparatively little is known about such proteins in plants. DRMs are thought to be assembled at the TGN on their way to the PM (Klemm et al., 2009), but DRMs have also been associated with subdomains of the ER including mitochondria-associated membranes and regions specialized in ER quality control (Lynes and Simmen, 2011). Given the large amount of tonoplast membrane that needs to be synthesized before and during cell expansion, direct delivery of lipids and proteins from a subdomain of the ER to the tonoplast could provide a fast and efficient solution.

The Tonoplast: Where Does It Come from?

The ER as the origin of the tonoplast was proposed previously for different cell types in both monocots and dicots (Matile and Moor, 1968; Berjak, 1972; Hilling and Amelunxen, 1985; Staehelin, 1997). It was suggested that vacuoles are generated from smooth ER-derived membrane tubules that will fuse into relatively flat sacks eventually dilating to form small vacuoles. Furthermore, continued fusion of small vacuoles with nearby ER tubules and further dilation is supposed to result in the formation of larger vacuoles (Amelunxen and Heinze, 1984).

A second model describes a very similar sequence of events leading to the formation of larger vacuoles; however, here the TGN is postulated to be the membrane source (Marty, 1978). 3D high-voltage EM of mitotic cells in the horseradish root revealed the presence of a complex tubular network. Proximity of nascent provacuoles to nodes of the TGN combined with the fact that both compartments are acidified and contain acid hydrolases was taken as evidence that provacuoles are derived from the TGN. Interestingly, according to both models, autophagy is important in the process of vacuole formation as they predict that portions of cytosol that are entrapped by the vacuolar network need to be degraded. We cannot fully exclude this, but our data provide two major arguments against a contribution of autophagy.

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First, although in two-dimensional images areas of cytosol indeed appear to be trapped in the developing vacuole (Figure 4B; see Supplemental Figures 4B and 4C online), 3D visualization (Figures 4E to 4H) of the vacuolar network shows that cytosol and vacuolar lumen form two continuous and separate spaces. Second, we have shown here that the core autophagy machinery that is involved in all subtypes of macroautophagy (Xie and Klionsky, 2007) is not required for vacuole biogenesis. This is of particular interest, as the structure of provacuoles and autophagosomes is highly similar. Both are characterized by the presence of a double membrane and their spherical appearance. In contrast with the autophagosome, which is a complete sphere formed by fusion of the phagophore, provacuoles often appear cup shaped, indicating that their rims do not fuse. The second distinctive feature is that autophagosomes become acidic only after fusion with lysosomes or small vacuoles (Klionsky et al., 2008), whereas the high density of proton pumps is characteristic of provacuoles. Formation of phagophores remains controversial, and it seems that multiple sources are involved (Tooze and Yoshimori, 2010) among which the ER seems to be the prime candidate (Hayashinishino et al., 2009, 2010). Our model for vacuole formation is similar to what has been proposed for phagophores, as it predicts that a subdomain of the ER devoid of ribosomes and with a distinctive lipid composition accumulates proteins and lipids destined for the tonoplast. After this membrane sheet reaches a certain size, it would curve (Knorr et al., 2012) and form the typical cup-shaped provacuole that will eventually fuse with the preexisting vacuolar network (Figure 8D). The fact that multilamellar provacuoles are formed when post-Golgi trafficking is blocked could indicate that a component delivered by the TGN is required either for separation of the provacuole from the ER or for fusion with the vacuolar network. Post-Golgi trafficking is clearly required for later stages of vacuole formation as well as for vacuolar function, and it also remains to be determined if the ER continues to directly deliver material once a large central vacuole has been established. In conclusion, our data provide strong evidence for a Golgi-independent route of vacuolar biogenesis in plant cells. Our findings thus open perspectives for future analyses of the molecular mechanisms underlying vacuole biogenesis and the related trafficking pathways.

METHODS

Plant Materials and Growth Conditions

Plants expressing VHA-a3-GFP or VHA-a3-mRFP were previously described (Dettmer et al., 2006; Brüx et al., 2008). The gnl1 mutant and the
GNL1-Myc BFA-sensitive (GNL1-BFA) line in the gnl1 background were previously established (Richter et al., 2007). The atg2, atg5, and atg7 mutant lines have been described previously (Thompson et al., 2005; Inoue et al., 2006; Hofius et al., 2009). The post-Golgi mutant lines pat2 and amsh3 and the transgenic line expressing si/VPS4S_10d have been previously described (Zouhar et al., 2009; Feraru et al., 2010; Isono et al., 2010). Arabidopsis thaliana seedlings ecotype Columbia-0 (Col-0) were grown on 1× Murashige and Skoog (MS) medium and 1% Suc with pH adjusted to 5.8 with potassium hydroxide (KOH). Plates were solidified using 0.6% phyto agar. Agar and MS basal salt mixture were purchased from Duchefa. Seeds were surface sterilized with ethanol followed by stratification for 48 h at 4°C. Seedlings were grown at 22°C with cycles of 16 h light and 8 h darkness. For vacuolar pH measurements, seeds of Arabidopsis ecotype Col-0 seeds were surface sterilized with ethanol and sown on plates containing 0.5× MS, 0.5% Suc, and 2 mM MES-KOH, pH 5.8, solidified with 0.5% phyto agar.

Pharmacological Treatments and Stains

Five-day-old seedlings were incubated in 0.5× MS liquid medium with 0.5% Suc, pH 5.8, containing 1 μM ConA, 50 μM BFA, 10 μM BCEF, 10 μM SNARF-1, 2 μM FM4-64, and 50 μM CHX or the equivalent amount of DMSO in control samples for the indicated time and combinations at room temperature. Stock solutions were prepared in DMSO.

Constructs

The INT1 coding sequence was amplified using the primers INT1-Gateway-F and INT1-Gateway-R. The resulting PCR fragment was introduced into pENTR/D-TOPO for sequencing, and the resulting plasmid was named pSW47. Afterwards, the INT1 sequence was introduced into the destination vector pK7FWG2.032. A positive clone (pSW48) was used for transformation of the agrobacterial stain C58C1, and the resulting stain was named A-pSW48. Stably transformed plants (Col-0) were generated using the floral dip method (Clough and Bent, 1998).

The coding sequence of At5g52980 was amplified from Col-0 cDNA using primers At5g52980.FOR and At5g52980.REV. The fragment was ligated into the Xmal-Sacll-digested pURT2kan, which is a derivative of the pH212 vector containing the UB100 promoter and the open reading frame of mRFP, resulting in VMA12-mRFP. A positive clone was used for transformation of the agrobacterial strain C58C1, and the resulting strain was named A-pSW48. Stably transformed Arabidopsis plants (Col-0) were generated using the floral dip method (Clough and Bent, 1998).

FRAP

Arabidopsis seedlings expressing VHA-a3-mRFP (in gnl1, GNL1-Myc BFA) were first incubated for 3 h in BFA and then mounted in BFA-supplemented 0.5× MS medium onto a Leica TCS SP5II microscope equipped with a HCX PL APO CS 20.0 × 0.70 mm UV objective. The region of interest was selected and bleached to a fluorescence intensity of ~50% compared with the unbleached region by scanning three times using a laser intensity of 100%. The scan parameters were as follows: image dimension, 1024 × 1024; pinhole, 1 airy unit; line average, 5. For prebleach and postbleach image acquisition, laser power was switched back to standard settings and recorded at the indicated time points. Leica Application Suite Advanced Fluorescence software was used for quantitative analysis. To avoid errors due to lateral diffusion of fluorescent proteins on the tonoplast membrane in partially bleached cells, only cells at the center of the bleached region were taken into account. The mean fluorescence intensity ratio of unbleached and bleached regions was then calculated to measure the synthesis of new VHA-a3-mRFP.

Vacuolar pH Measurements

Vacuolar pH measurements in Arabidopsis roots were essentially done as described before (Krebs et al., 2010) with some minor modifications. Five-day-old seedlings were incubated in liquid medium (0.5× MS, 0.5% Suc, and 2 mM MES-KOH, pH 5.8) containing 10 μM membrane-permeant pH-sensitive dye BCECF-AM (Molecular Probes, Invitrogen) and 0.02% Pluronic F-127 (Molecular Probes, Invitrogen). Dye loading was conducted for 1 h at 22°C in darkness followed by two washing steps, 5 min each, in liquid medium. Fluorescence microscopy was performed on a Leica SP5II confocal laser scanning microscope equipped with an inverted DMi6000 microscope stand and a HCX PL APO ×63 water immersion objective. BCECF was excited with 458 and 488 nm, and the emission was detected between 510 and 550 nm. To exclude background fluorescence and oversaturated pixels, images were thresholded using ImageJ v 1.46 (National Institutes of Health). The average fluorescence intensities were measured in defined regions of interest as indicated in Supplemental Figure 2 online, followed by calculating the ratio between the intensities of the 488- and 458-nm excited images. In vivo calibration was done by treating BCECF-loaded seedlings for 20 min with pH equilibration buffers containing 50 mM MES-Bis-tris propane (pH 5.2 to 6.4) or 50 mM HEPES-Bis-tris propane (pH 6.8 to 8.0) and 50 mM ammonium acetate. Ratio values were plotted against the pH, and the calibration curves were generated using a sigmoidal Boltzmann fit.
Filipin-Sterol Fluorescence Microscopy and Ultrastructural Analyses of Filipin-Sterol Complexes

Labeling for filipin-sterol fluorescence on pVHAa3:VHA-a3-GFP–expressing seedlings and fluorescent anti-KNOLLE/filipin-sterol colabeling in wild-type Col-0, gn1−1, GNL1 BFA-resistant, and GNL1-BFA2 lines were performed as described (Boutté et al., 2010). Antibodies dilutions were as follows: rabbit anti-KNOLLE, 1:4000 (Lauber et al., 1997); Cy5-coupled, donkey anti-rabbit IgG, 1:300 (Jackson Immunoresearch). Fluorescence was detected by CLSM using a Leica TCS SP2 AOBS spectral system mounted on a Leica DM IRE2 inverted microscope equipped with a 364-nm argon UV laser. Image acquisition settings were as described (Boutté et al., 2010). Sequential line-scanning mode with a line average of 8 was used during multiple labeling acquisition. An oil-corrected ×63 objective, numerical aperture of 1.4 (HCX PL APO 63.0_1.40 OI BD UV; Leica) was used.

During multiple labeling acquisition. An oil-corrected ×63 objective, numerical aperture of 1.4 (HCX PL APO 63.0_1.40 OI BD UV; Leica) was used. EM of the INT1-GFP line. M.G. planned sterol localization experiments. C.V. and K.S. planned the project. C.V., F.K., C.N., F.F., U.L., M.K., D.S., Y.B., M.F.-R., and S.H. carried out the experiments. S.W. and N.S. established the INT1-GFP line. M.G. planned sterol localization experiments and performed EM on filipin-treated samples. All authors analyzed and discussed the data. K.S. wrote the article. All authors read and commented on the article.

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AUTHOR CONTRIBUTIONS

C.V. and K.S. planned the project. C.V., F.K., C.N., F.F., U.L., M.K., D.S., Y.B., M.F.-R., and S.H. carried out the experiments. S.W. and N.S. established the INT1-GFP line. M.G. planned sterol localization experiments and performed EM on filipin-treated samples. All authors analyzed and discussed the data. K.S. wrote the article. All authors read and commented on the article.

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