Tethering complexes HOPS/CORVET are required for vacuole integrity

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1 Introduction

Lytic vacuoles are one of the most characteristic organelles in plant cells with numerous vital functions in growth and development. By investigating the trafficking of vacuolar proton pumps (V-ATPase and V-PPase) in meristematic root cells of *Arabidopsis thaliana*, we could recently show that *de novo* formation of lytic vacuoles takes place directly at the endoplasmic reticulum (ER). Transport of proton pumps to the vacuole bypasses Golgi, TGN/EE and MVB/LE compartments but occurs via provacuoles. [1] However, mechanistic knowledge is missing so far. Our current research aims to understand how and which factors promote the formation of provacuoles at the ER. If we assume that provacuoles are formed constantly at the ER but are not a continuosly connected with the vacuole, these structures have to fuse with the already existing vacuolar network. These fusion events would require a membrane tethering complex like HOPS or CORVET.
A promising candidate in this regard is VACUOLELESS1 (VCL1). Its homozygous knockout mutant does not develop discernable vacuoles but is embryo lethal. VCL1 is a yeast VPS16 homolog.[2] VPS16p is a shared but not essential subunit of the membrane tethering complexes HOPS and CORVET, thereby promoting the fusion of endosomes with vacuoles and lysosomes. In yeast and mammals CORVET complexes enable fusion between the trans-Golgi network and early endosomes as effector proteins of Rab5-GTPases. As the early endosomes mature to late endosomes, a Rab5-to-Rab7 conversion occurs to recruit HOPS complexes (as Rab7-GTPase effectors) in order to fuse with lysosomes or vacuoles. [3] In Arabidopsis, the early endosome and trans-Golgi network are the same compartment. [4] Still, late endosomes mature from this compartment [5] and need to undergo a Rab5-to-Rab7 conversion in order to fuse with the vacuole. As in yeast and mammals, this conversion is mediated by the SAND1/Mon1 complex. [6 - 8]

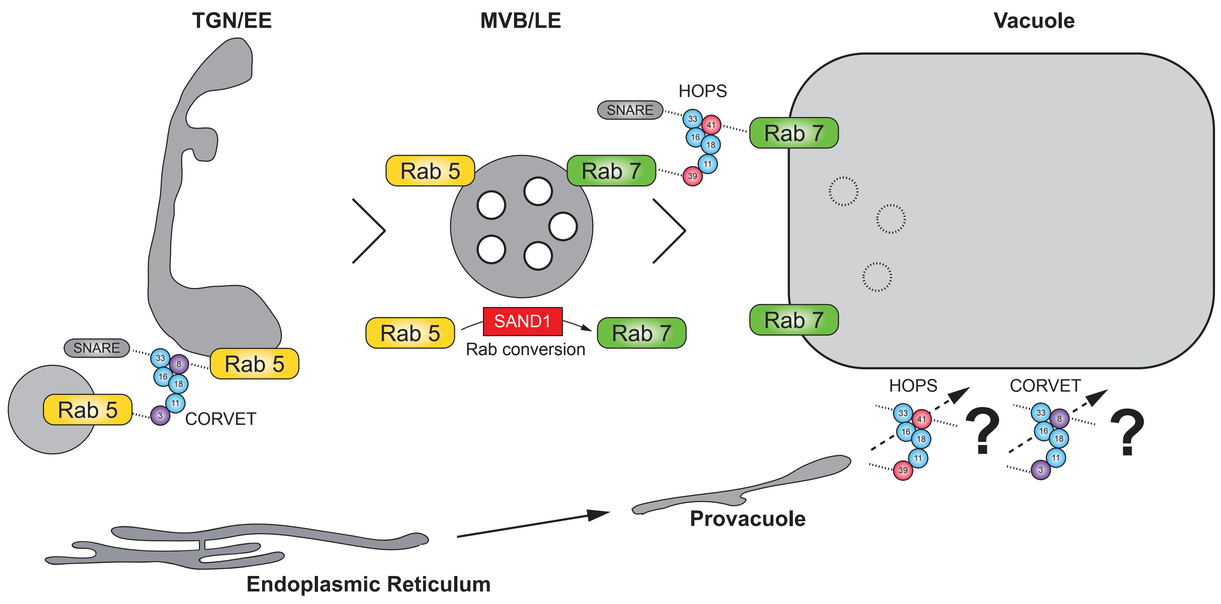


Figure 1: Schematic representation of endosomal compartments that directly contribute to vacuolar trafficking, the corresponding Rab-GTPases and tethering complexes. The trans-golgi network/early endosome (TGN/EE) has subdomains carrying Rab5-GTPases that mature into multivesicular bodies/late endosomes (MVB/LE). Before MVB/LEs fuse with the vacuole, they undergo a Rab5-to-Rab7 conversion carried out by SAND1. Rab7-GTPases recruit HOPS complexes that tether late endosomal and vacular membrane and simultaneously coordinate SNARE proteins. Finally, this leads to the fusion of MVBs with the vacuole. This principle is the same for yeast and mammals with the exception that early endosomes and TGNs are seperate compartments that require CORVET complexes in order to fuse. *De novo* formation of vacuoles in *Arabidopsis thaliana* occur via provacuoles that eventually fuse with th existing vacuole but it is not known if HOPS or CORVET are required for that, too.

2 Function of HOPS and CORVET

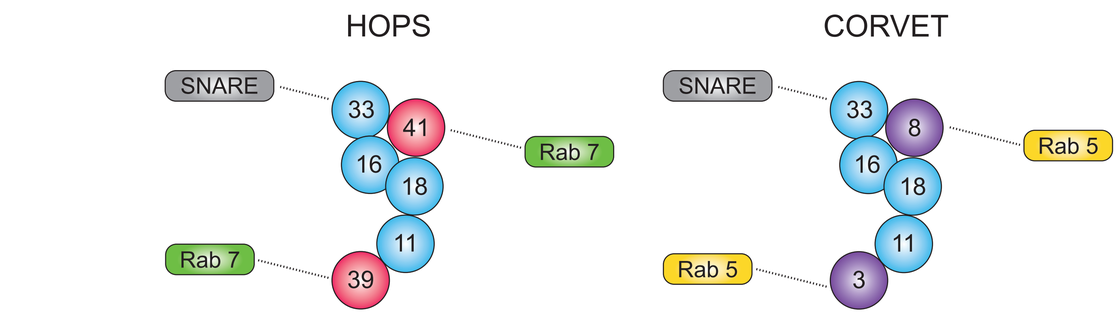
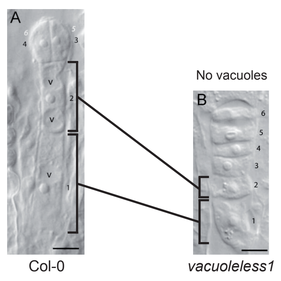


Figure 2: Schematic model of HOPS and CORVET showing the individual subunits that are shared (blue) specific (red or purple) and the proteins they bind to (green, yellow and grey). Modified from [9]

The transport of cargo proteins within the endosomal membrane system is carried out mostly by vesicles. To ensure the correct localization of their cargo, vesicles need to fuse solely with their target membrane. HOPS and CORVET are two hetero-hexameric protein complexes that specifically tether donor and acceptor membranes, bring SNARE proteins in position and thereby promoting fusion. Both complexes share 4 similar subunits (Figure 2, above) but their specificity is established by the different Rab GTPases they interact with.
It was shown in yeast and mammals that CORVET interacts with Rab5 GTPases, whereas HOPS forms complexes with Rab7 GTPases. These preferences are provided by the specific subunits VPS39 and VPS41 for HOPS and VPS8 and VPS3 for CORVET. [9] According to the yeast model, *Arabidopsis* homologs can be identified for both, the shared and the specific subunits. To distinguish between HOPS and CORVET, VPS8 and VPS39 are the most promising candidates based on sequence similarity.



VACUOLELESS1 encodes a class-C VPS protein homologous to VPS16p of *S.cerevisae*. In yeast, it was shown to be part of both the HOPS and CORVET membrane tethering complex that is required but not essential for the biogenesis of and trafficking to the vacuole.
In contrast, the homozygous *vacuoleless1* (*vcl1*) knockout mutant in *A.thaliana* is embryonic lethal. This mutant was identified in a T-DNA screen for defects in early embryonic development and vacuole morphology. *vcl1* embryos have no discernable vacuoles (Figure 3, on the left) and fail to elongate as well as properly divide cells. In addition, localization studies by immunocytochemistry showed that VCL1 localizes to MVB/LEs. As shown in yeast, VCL1 can interact with VPS11 and VPS33 and is therefore assumed to be part of the HOPS vacuolar membrane tethering complex in Arabidopsis. [2, 10]

Figure 3: 4-cell stage embryo of Col-0 and *vcl1*. Modified from [2]

3 GFP-VCL1 localizes to early and late endosomes

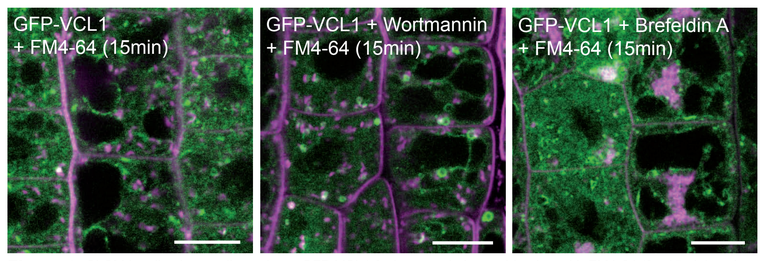


Figure 4: CLSM localization studies using a GFP-VCL1 fusion protein in combination with FM4-64 (for 15min) and additional inhibitors of endosomal trafficking Wortmannin and BFA. Scale bars indicate 10µm.

It has been shown by immunocytochemistry that VCL1 is localized at prevacuolar compartments (late endosomes) and tonoplast. [10] We wanted to confirm this by using a GFP fusion protein driven by a Ubiquitin10 promoter. As expected, GFP-VCL1 shows a cytosolic signal.
In addition, there are punctae that partially colocalize with FM4-64 staining after 15min and can be considered as TGN/EE compartments. After BFA treatment only a subpopulation is trapped in the induced hybrid compartments. The second population of punctae is sensitive to wortmannin, identifying them as late endosomes.
Interestingly, tonoplast signal can only be observed in limited patches.

4 Knockdown of *VCL1* causes strong developmental phenotypes

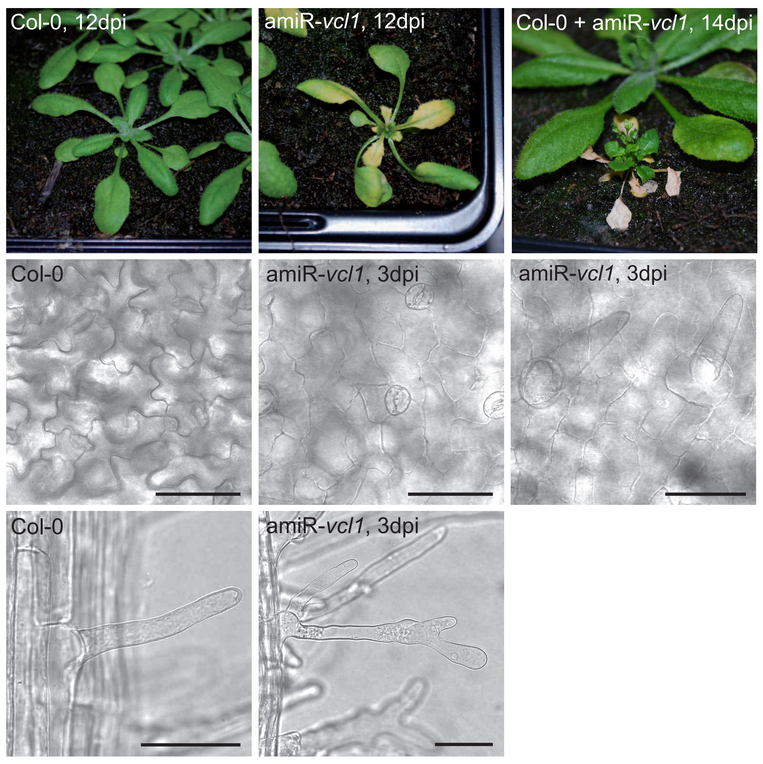
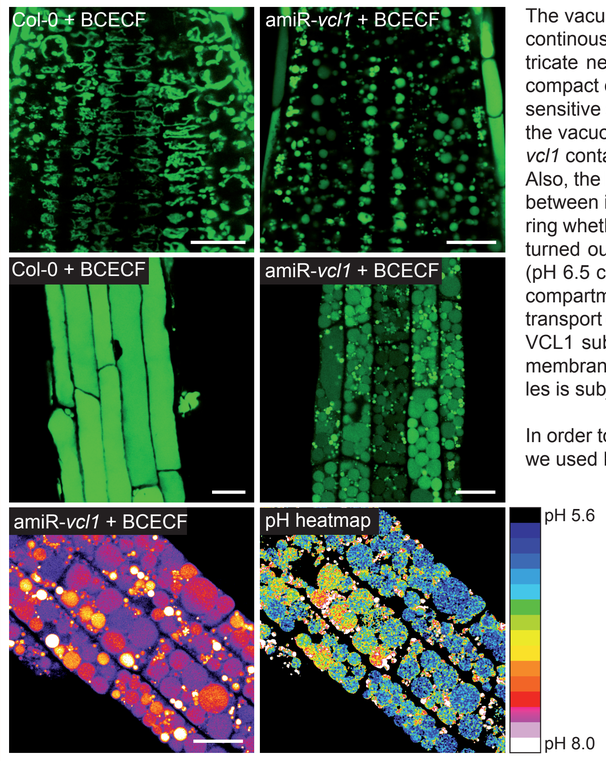


Figure 5: Summary of macroscopic phenotypes of induced VCL1 knockdown plants at different timepoints after Dexamethasone (DEX) treatment. To induce the expression of amiR-*vcl1* the plants are simply sprayed once with 30µM DEX dissolved in water and observed at a given day after induction (dpi). Seedlings are incubated in 1/2MS medium supplemented with DEX. Scale bars indicate 50µm.

One way to study the function of proteins in plants is to investigate corresponding T-DNA knockout mutants. In the case of *VACUOLELESS1,* a homozygous knockout mutant is embryo lethal. Therefore, we designed an artificial micro-RNA (amiR) against *VCL1* using the Web MicroRNA Designer (WMD3). In order to control the timepoint of VCL1 knockdown we combined the amiR-*vcl1* construct with an Dexamethazone (DEX)-inducible expression system using GreenGate cloning. Without induction of amiR-*vcl1* expression during embryo development we can avoid lethality. This allows us to study the effect of reduced VCL1 expression in the meristematic root zone of Arabidopsis and address the question of how VCL1 as a member of HOPS/CORVET contributes to the biogenesis of lytic vacuoles.
Compared to wild type plants of the same age and treatment, amiR-*vcl1* plants exhibit strong developmental phenotypes that are very similar to several different microtubule defective mutants. (Figure 5) Instead of forming straight petioles, petioles of knockdown plants twist in a left-handed manner. The twisted aerial organs perish soon and the residual plant shows severe growth retardation. It has to be stated that these plants are not delayed in flowering. A closer look at cotyledons revealed that epidermal cells are impaired in proper lobe formation. Additionally, trichomes do neither elongate nor branch.
The opposite happens with root hairs. Instead of a single tip outgrowth, root hairs of seedlings expressing amiR-vcl1 often split several times.
With those observations we were curious to see if the subcellular defects are comparibly severe.

5 Integrity and development of vacuoles is dependent on VCL1

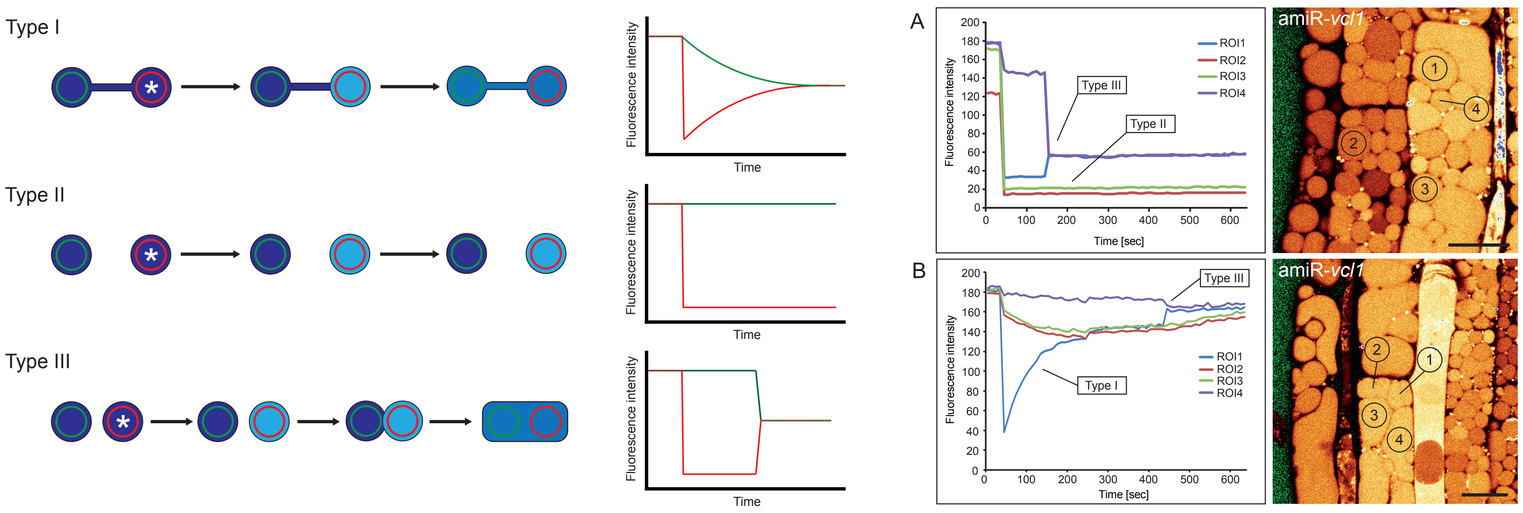


The vacuolar lumen in meristematic cells of *Arabidopsis thaliana* root cells is a single continous tubular entity that inflates over time as the cell matures. In contrast to the intricate network of young cells, vacuoles of fully differentiated cells appear as huge compact organelles that can take up more than 90% of the cell volume. Using the pH sensitive dye BCECF that is efficiently taken up into lytic compartments one can stain the vacuolar lumen *in vivo*. Compared to wildtype vacuoles, cells that express amiR-*vcl1* contain vacuoles that appear to be spherical and highly fragmented.
Also, the loading with BCECF seems to be uneven as the intensity differs significantly between individual cells and vacuoles. Since BCECF is pH sensitive we were wondering whether the intensity differences are due to the altered pH or loading issues. As it turned out, the average pH in vacuoles cells expressing amiR-*vcl1* is more alkaline (pH 6.5 compared to pH 5.8 in Col-0). Surprisingly, we could also observe individual compartments with neutral or even more alkaline pH values suggesting an impaired transport of proton pumps to the vacuole. One could speculate that due to the missing VCL1 subunit non-functional HOPS/CORVET complexes cannot tether provacuolar membrane with the tonoplast. But whether the observed compartments are still vacuoles is subject of furhter investigations.

In order to determine if the spherical vacuoles are really isolated or are still in contact, we used Fluorescence Recovery After Photobleaching (FRAP).

Figure 6: CLSM studies investigating the subcellular phenotype of vacuoles in cells of seedlings expressing amiR-*vcl1*. To stain the vacuolar lumen we incubated the seedlings with 10µM BCECF (2h) and washed them (1h) before imaging. Note the different vacuole morphologies in uninduced and induced cells. In order to calculate pH values with BCECF ratio images (488/458), a calibration curve was measured. Scale bars indicate 20µm.

6 Using FRAP to analyse vacuolar connections



The induced knockdown of VCL1 has a severe impact on vacuole integrity, leading to the many described developmental phenotypes. Assuming that the inducible knockdown of VCL1 expression is not absolute, we would expect to have still functional HOPS and CORVET complexes that can promote fusion between vacuoles, other vacuoles and provacuoles. To determine whether the vacuoles that seem to be fragmented are indeed isolated and unable to fuse, we applied the Fluorescence Recovery After Photobleaching (FRAP) method with BCECF loaded vacuoles of induced seedlings.
Depending on their possible types of connection (Type I to III) and the amount of unbleached BCECF motion within the vacuolar lumen, the fluorescence will recover differently. While a limited tubular connection will slowly homogenize the fluorescence intensity between bleached and unbleached vacuole (Type I), isolated vacuoles will not recover at all (Type II). If isolated vacuoles happen to fuse, the fluorescence intensity should immediately equalize between the fusion partners.
Our measurements consistently show that we find all three vacuole connection types, meaning that there are both still connected and isolated vacuoles. Most of the isolated vacuoles were shown to be still fusion competent. What we do not know yet is, if the vacuoles really do fragment (brake apart) or if newly synthesized provacuoles can no longer fuse with the existing vacuoles.

7 Conclusion and outlook

The work presented on this poster shows that:

- GFP-VCL1 is localized at the TGN/EE, MVB/LE and restricted patches at the tonoplast
- the induced expression of amiR-*vcl1* avoids embryo lethality of *vcl1* mutants
- VCL1 knockdown leads to severe growth and developmental defects similar to known microtubule mutants
- the HOPS/CORVET complex is required for vacuole integrity

As a last remark it should be stated that the microtubule-related phenotypes may suggest a crucial role of vacuoles acting as counterpart to the cytoskeleton.
Our ongoing work aims at the question what exact tethering complex is involved in vacuole integrity and how this connects to the formation of provacuoles. All of this as part of how vacuole biogenesis occurs mechanistically.

8 References

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