Distinct Lytic Vacuolar Compartments are Embedded Inside the Protein Storage Vacuole of Dry and Germinating Arabidopsis thaliana Seeds

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Plant cell vacuoles are diverse and dynamic structures. In particular, during seed germination, the protein storage vacuoles are rapidly replaced by a central lytic vacuole enabling rapid elongation of embryo cells. In this study, we investigate the dynamic remodeling of vacuolar compartments during Arabidopsis seed germination using immunocytochemistry with antibodies against tonoplast intrinsic protein (TIP) isoforms as well as proteins involved in nutrient mobilization and vacuolar acidification. Our results confirm the existence of a lytic compartment embedded in the protein storage vacuole of dry seeds, decorated by γ-TIP, the vacuolar proton pumping pyrophosphatase (V-PPase) and the metal transporter NRAMP4. They further indicate that this compartment disappears after stratification. It is then replaced by a newly formed lytic compartment, labeled by γ-TIP and V-PPase but not AtNRAMP4, which occupies a larger volume as germination progresses. Altogether, our results indicate the successive occurrence of two different lytic compartments in the protein storage vacuoles of germinating Arabidopsis cells. We propose that the first one corresponds to globoids specialized in mineral storage and the second one is at the origin of the central lytic vacuole in these cells.

Keywords: Arabidopsis • Germination • Seed • Vacuole.

Abbreviations: DIC, differential interference contrast; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; IgG, immunoglobulin G; mRab, myristoylated Ras-like proteins in brain; NRAMP, natural resistance-associated protein; PBS, phosphate-buffered saline; PSV, protein storage vacuole; SNARE, soluble NSF attachment protein receptor; Syp, syntaxin of plants; TIP, tonoplast intrinsic protein; V-ATPase, vacuolar ATPase; V-PPase, vacuolar pyrophosphatase.

Introduction

The common textbook image of a single vacuole carrying multiple functions has been refined over the past few decades by cell biologists and physiologists, resulting in the hypothesis of the existence of multiple functionally distinct vacuoles in plant cells (Marty 1999). Vacuole functions include the storage of proteins, inorganic ions, other nutrients and secondary metabolites, detoxification of heavy metals, salt and xenobiotics, pH homeostasis and turgor maintenance. Protein storage vacuoles are most abundant in seeds, whereas the storage of secondary metabolites, detoxification, pH homeostasis and turgor maintenance are mainly accomplished by large central vacuoles in vegetative tissue (Martinoa et al. 2007). In this context, the use of different sets of tonoplast intrinsic proteins (TIPs) and cargo proteins such as storage proteins and proteases has helped to establish the existence of at least two functionally distinct vacuoles, protein storage vacuoles (PSVs) and lytic vacuoles (Neuhaus and Rogers 1998). Paris and co-workers showed that, in root tips of barley and pea seedlings and in mature tobacco plants, two distinct vacuoles coexist in the same cell, one containing α-TIP on the tonoplast and lectin as cargo called the PSV and another defined by γ-TIP on the tonoplast and the protease aleurain as cargo called the lytic vacuole (Paris et al. 1996). In Arabidopsis thaliana, α-TIP and γ-TIP, two aquaporins, correspond to TIP3;1 and TIP1;1 to 1;3, respectively (Beebo et al. 2009, Wudick et al. 2009).

The two TIP isoforms have been routinely used as markers for vacuolar functions with α-TIP typically found in seed PSVs, and with γ-TIP being used to identify lytic vacuoles (Jauh et al. 1999). It has already been reported that the real situation is much more complex, since in seeds the PSV labeled by α-TIP might carry γ-TIP-positive globoids inside their lumen and that...
in root tips γ-TIP and α-TIP labeling may co-localize in certain regions of the vacuolar compartment on the tonoplast in cells that develop large central vacuoles (Paris et al. 1996, Jiang et al. 2001). Also, cells may contain two large central vacuoles exhibiting differential functions such as accumulation of salt or malic acid in *Mesembryanthemum crystallinum*, and accumulation of secondary metabolites, compared with vacuoles responsible for rapid water loss in the pulvini of *Mimosa pudica* (Martinoia et al. 2007). Recently, the co-existence of multiple functionally distinct vacuoles and the clear-cut distinction between them has been questioned by several studies raising questions on the use of markers of the TIP family and of cargo molecules (Vitale and Hinz 2005, Hunter et al. 2007, Olbrich et al. 2007, Frigerio et al. 2008) in Arabidopsis and other plants, especially in seeds. It was suggested that the expression of α-TIP and γ-TIP was specific to tissue types and developmental stages rather than vacuole types and that two different vacuolar sorting signals targeted cargo to a single vacuolar location in Arabidopsis (Hunter et al. 2007).

Seeds provide a model of choice to study the transition between multiple functionally distinct vacuoles. During seed germination, storage proteins, which provide a source of reduced nitrogen, and inorganic minerals need to be mobilized to support seedling growth. In addition, a lytic aqueous vacuolar compartment has to be formed to provide turgor pressure driving cell expansion and to promote radicle protrusion and embryo elongation. It has been proposed that vacuolar membranes of PSVs are transformed into those of central vacuoles upon germination (Maeshima et al. 1994). Findings in soybean and pumpkin have shown that during germination the PSV undergoes an important dedifferentiation with gradual degradation of α-TIP, internalization of the tonoplast and formation of a new tonoplast carrying vacuolar H^+-ATPase, V-PPase and γ-TIP (Maeshima et al. 1994, Inoue et al. 1995). These results suggested that during germination, the dynamic changes in the vacuole rapidly lead to the formation of lytic compartments.

In the present work, we studied vacuolar remodelling in Arabidopsis upon germination by carrying out immunocytochemistry with a set of vacuolar and prevacuolar markers. To identify the PSV and lytic vacuolar compartments, we used α-TIP and γ-TIP, respectively. In view of the ambiguity of these markers, we furthermore used other markers directly related to lytic vacuole functions such as vacuolar H^+-ATPase and V-PPase that promote vacuolar acidification (Maeshima et al. 1994) and NRAMP4, a metal transporter that functions in iron retrieval from the seed vacuoles (Lanquar et al. 2005). Secondly, we used TIP1:2:GFP-expressing plants (Beeko et al. 2009) to increase the specificity of TIP isoform recognition, since the γ-TIP antibodies used in the earlier studies may not have been specific enough to discriminate between close TIP homologs. Thirdly, we performed uptake of the vital stain Neutral Red, a fluorescent dye trapped inside acidic compartments upon protonation, to identify acidic compartments. Finally, we monitored the labeling with the prevacuolar marker mRab, and the SNARE Syp21, both involved in protein transport between the Golgi apparatus and the lytic vacuole (Sanderfoot et al. 2001, Bolte et al. 2004, Foresti et al. 2006).

We show that γ-TIP and NRAMP4 label similar structures embedded in the PSV of dry seeds, whereas their labeling patterns diverge upon germination. γ-TIP and α-TIP are present in the same cell, but label distinct membranes in dry, stratified and germinated seeds. In germinated seeds, we observe a newly formed compartment inside the PSV. This compartment is strongly labeled by γ-TIP, vacuolar H^+-ATPase and pyrophosphatase but not NRAMP4. Furthermore, prevacuolar compartments (PVCs) labeled by mRab and Syp21 are found at the periphery between the PSV and the newly formed acidic compartment. Concomitantly, an acidic compartment accumulating Neutral Red in vivo is formed. Our results illustrate the complex remodelling of the vacuome upon germination.

**Results**

**Morphological modifications of the PSV during germination**

To investigate the dynamics of seed vacuoles during germination, three different stages were examined: dry seeds, stratified seeds and seeds germinated for 1 d in the light (see Material and Methods). To avoid labeling differences due to heterogeneous cell types, the same defined region of the embryo axis, corresponding to the hypocotyl cells next to the radicle, was consistently observed throughout the study (Fig. 1A–C, arrowheads). The content of PSVs was revealed by their autofluorescence (Fuji et al. 2007, Hunter et al. 2007) (Fig. 1A–C, D). A section through the plane of the nucleus (Fig. 1D–D') shows that the autofluorescence (blue color) labels specifically the PSV and is not present in the nucleus or the cell wall. In dry seeds, PSVs are round-shaped structures of 5–10 μm diameter containing 2–4 μm embedded structures that are devoid of autofluorescence (Fig. 1A, A', arrow). In stratified seeds, the size of the PSV expands and the PSV content becomes heterogeneous (Fig. 1C, C', open arrowhead). We first investigated the localization of the two markers NRAMP4 and γ-TIP in dry seeds and their fate during germination. Then, we compared their labeling patterns with that of the specific PSV marker α-TIP at three stages of germination.

γ-TIP and NRAMP4 label similar structures in dry and stratified seeds of *A. thaliana* but distinct structures in germinating seeds

In dry seeds NRAMP4 and γ-TIP labeling is concentrated at the periphery of the structures lacking autofluorescence embedded inside the PSV described in Fig. 1 (Fig. 2A, D, arrows). In addition, NRAMP4 localizes to the tonoplast surrounding the PSV (Fig. 2A). In stratified seeds, both markers show mainly
Fig. 1  Morphology of Arabidopsis seed sections used for immunolabeling studies. Overview (A–C) and magnification (A′–C′, A″–C″, D–D″) of sections revealed by autofluorescence (A′–C′, D) and DIC (A–C, A″–C″, D′, D″), of dry (A, A′, A″, D, D′, D″), stratified (B, B′, B″) and seeds that were germinated for 24 h (C, C′, C″). DIC images A′–D″ were merged with autofluorescence images. Scale bars are 10 μm except (A–C) 100 μm. All confocal and widefield images shown in the following figures were taken from cells located in the embryo axis (solid arrowhead) on longitudinal sections of the Arabidopsis embryo. Autofluorescence at 405 nm (A′–C′) is mainly present in PSVs as revealed by DIC imaging (A″–C″). Cross-sections through the nucleus (D, D′, D″) confirm that autofluorescence is present in the PSV (psv) but absent in the nucleus (n) and the cell wall (arrows in D and D″). In the following figures, autofluorescence was used as counterstaining to label the position of PSVs. PSV-embedded structures (arrows) in dry seeds become smaller during stratification. Twenty-four hours after germination cavities of heterogeneous size appear in the PSV (asterisks) that are sometimes connected with the cytosol (open arrowhead).
discontinuous labeling around the PSV (Fig. 2B, E). In addition, both markers label some punctate cytosolic structures (Fig. 2B, E; arrowheads) and some labeling of structures embedded in the PSV observed in dry seeds eventually remains visible in stratified seeds (Fig. 2B, arrows). In germinated seeds NRAMP4 labels punctate submicron cytosolic structures (Fig. 2C). In contrast, γ-TIP labels mainly the edge of the cavities inside the PSV (Fig. 2F, asterisk). This observation hints at the possibility that the cavities correspond to a newly formed lytic vacuole inside the PSV in germinating seeds. We then performed α-TIP labeling to study the distribution of this PSV marker at the three stages of seed germination.

**α-TIP localization is distinct from γ-TIP and NRAMP4 localization in dry seeds and germinating seeds**

α-TIP has been reported to be present on the tonoplast of the PSV until 2.5 d after germination of Arabidopsis seeds using fluorescent fusion proteins (Hunter et al. 2007). Immunolabeling of seed sections corroborates this finding: α-TIP labeled preferentially the tonoplast of the PSV at all stages examined: dry seeds, stratified seeds and germinated seeds (Fig. 2G–I). Additionally, in germinated seeds α-TIP labels some smaller adjacent vacuoles in addition to the PSV (Fig. 2I, open arrow).

**Specific immunolabeling of TIP1;1, a γ-TIP, in germinating seeds using plants expressing TIP1;1::GFP under the control of TIP1;1 native promoter**

Based on the observation of TIP1;1::GFP fusion protein fluorescence, Hunter and co-workers reported that TIP1;1 is not present in Arabidopsis seedlings during early seed germination and appears only 3.5 d after germination when it replaces α-TIP (Hunter et al. 2007). TIP1;1 is the major isoform of γ-TIP and our observations using γ-TIP antibodies show labeling during early seed germination (Fig. 2). We thus re-investigated the localization of TIP1;1 using immunolabeling. For this purpose, we have used TIP1;1 KO plants expressing TIP1;1::GFP under the control of the TIP1;1 native promoter (Beebo et al. 2009). Note that this construct complements the mutant phenotype indicating correct subcellular localization (Beebo et al. 2009). Expression under the native promoter ensures that the expression pattern is the same as for the endogenous TIP1;1 protein. We carried out immunolabeling of green fluorescent protein (GFP) to detect TIP1;1::GFP. This permitted the detection of TIP1;1 with high specificity while increasing the detection sensitivity compared with observation of GFP fluorescence.

Sections of transgenic dry, stratified or germinated seeds showed no visible GFP signal for TIP1;1, in agreement with the results of Hunter and co-workers (2007). However, after enhancement of the GFP signal, immunolabeling with an anti-GFP antibody revealed by a FITC-coupled secondary antibody, TIP1;1 could be detected at the three stages of seed germination investigated (Fig. 3A–C). The labeling pattern resembles very much the labeling obtained with γ-TIP antibodies in wild-type dry and germinated seeds: in dry seeds, PSV-embedded round-shaped structures were strongly labeled (Fig. 3A, arrow). In germinated seeds the TIP1;1::GFP labeling was observed at the periphery of the cavity inside the PSV (Fig. 3C, asterisk). In stratified seeds, however, we observed different labeling with γ-TIP antibody and the GFP antibody used to detect TIP1;1::GFP: whereas the γ-TIP antibody labeled both the periphery of the PSV and cytosolic punctate structures (Fig. 2E, arrowheads), TIP1;1::GFP was detected only in cytosolic punctate structures at this stage (Fig. 3B, arrowheads). This suggests that the γ-TIP antipeptide antibody labeled other TIP isoforms in addition to TIP1;1. In order to directly compare the TIP1;1 pattern with α-TIP labeling, we then performed double labeling with anti-GFP and α-TIP antipeptide antibodies.

**TIP1;1::GFP labels a newly formed compartment inside the PSV in germinating seeds**

The double labeling of TIP1;1::GFP-expressing seeds was performed with GFP antibody revealed by a FITC-coupled secondary antibody and α-TIP antipeptide antibody revealed by Cy3-coupled secondary antibody. The double labeling pattern shows that both proteins are expressed in the same cells and confirms their distinct subcellular localization (Fig. 3D–F). In dry seeds, TIP1;1 labels mainly PSV-embedded structures (Fig. 3D, green signal, arrow) while α-TIP labeling (Fig. 3D, red signal) lines the tonoplast at the periphery of the PSV (Fig. 3D, blue signal). In stratified seeds, punctate TIP1;1 labeling is often located in close vicinity with α-TIP-positive tonoplast (Fig. 3E, arrowheads). In germinated seeds, the cavity inside PSV is strongly labeled by the anti-GFP antibody revealing the presence of TIP1;1::GFP whereas α-TIP labels the periphery of the PSV (Fig. 3F, asterisk). The double labeling in germinated seeds clearly shows that the TIP1;1-positive cavity is located inside the α-TIP-positive PSV (Fig. 3F). To rule out cross-reaction artefacts we performed control immunolabeling experiments (Supplementary Fig. S2) and to demonstrate proper imaging settings we studied the green and red fluorescence signals by tracing fluorescence intensity profiles through singly labeled green, and red and double labeled structures (Supplementary Fig. S3). We propose that the cavity labeled by TIP1;1 is a newly formed vacuolar compartment. To confirm the lytic nature of the newly formed compartment labeled by TIP1;1, we carried out immunolabeling with additional markers for lytic vacuoles and in vivo staining with Neutral Red, a dye that accumulates in acidic compartments, at the same stage.

**The cavity inside the PSV labeled by TIP1;1::GFP is a lytic compartment**

It has been shown that vacuolar H⁺-ATPase and H⁺-pyrophosphatase accumulate concomitantly with γ-TIP during the
Fig. 2 NRAMP4, γ-TIP and α-TIP labeling in dry, imbibed and germinating seeds. NRAMP4 (A–C), γ-TIP (D–F) and α-TIP (G–I) immunolabeling patterns in dry (A, D, G), stratified (B, E, H) and germinated (C, F, I) seeds of *A. thaliana*. Immunofluorescence with whole protein.
reformation of the central vacuole in germinating pumpkin seeds (Maeshima et al. 1994). Likewise, their activities increased during germination, suggesting that they provide the acidic conditions essential to hydrolytic enzymes (Maeshima et al. 1994). We therefore investigated the localization of these proton pumps in germinating seeds.

We performed labeling experiments on dry, stratified and germinated wild-type seed sections with antibodies directed against subunit A of the vacuolar H\(^+\)-ATPase (Zhigang et al. 1996). The labeling pattern of ATPase A resembles the labeling pattern of \(\gamma\)-TIP in dry and stratified seeds (data not shown). In dry seeds ATPase A localizes to PSV-embedded structures (A, D, arrow). In stratified seeds, it labels punctate cytosolic vesicles (B, arrowheads). Germinated seeds show a strong ATPase A membrane labeling of the cavity inside the PSV (C, asterisk). Note that \(\gamma\)-TIP labels predominantly the tonoplast of the PSV in dry, stratified and germinated seeds.

We furthermore studied the compartmentalization of Neutral Red, a dye that becomes trapped upon protonation in acidic compartments. In epidermal cells of living seeds that had been germinated for 24 h, Neutral Red accumulated in the lumen of cavities inside the PSV, as indicated by the fluorescence of this dye (Fig. 5, asterisk). These data support the immunocytochemical evidence for the presence of an acidic compartment inside the PSV of seeds germinated for 24 h.

**Fig. 3** TIP1;1::GFP and \(\gamma\)-TIP labeling and co-localization in TIP1;1 KO plants. Anti-GFP (A–F, green signal) and \(\gamma\)-TIP immunolabeling (D–F, red signal) was performed in TIP1;1 KO transgenic plants expressing TIP1;1::GFP under the control of the TIP1;1 own gene promoter in dry (A, D), stratified (B, E) and germinated (C, F) seeds. Immunofluorescence with anti-GFP antibody (FITC-coupled secondary anti-mouse antibody, green channel) and \(\gamma\)-TIP anti-peptide antibody (Cy3-coupled secondary anti-rabbit antibody, red signal) and auto-fluorescence (blue channel) images were superimposed; scale bars are 10 \(\mu\)m. Note that no GFP signal was observed prior to immunolabeling in dry, stratified and germinated seeds (data not shown). In dry seeds TIP1;1::GFP localizes to PSV-embedded structures (A, D, arrow). In stratified seeds, it labels punctate cytosolic vesicles (B, E, arrowheads). Germinated seeds show a strong TIP1;1 membrane labeling of the cavity inside the PSV (C, F, asterisk). Note that \(\gamma\)-TIP labels predominantly the tonoplast of the PSV in dry, stratified and germinated seeds.
The periphery of the lytic cavity inside the PSV is marked by prevacuolar markers

To investigate the membrane trafficking event associated with the formation of the acidic compartment observed inside the PSV of germinated seeds, we investigated two prevacuolar markers, Syp21, a syntaxin that is part of a SNARE complex involved in Golgi apparatus to PVC trafficking (da Silva Conceicao et al. 1997, Sanderfoot et al. 1998) and mRab, a small G-protein of the rab family that has been shown to be implicated in trafficking between the Golgi apparatus and the

Fig. 4 Vacuolar and prevacuolar marker localization in TIP1;1 KO plants. Vacuolar ATPase staining (A), pyrophosphatase staining (B), Syp21 labeling (C) mRab labeling (D) were revealed by FITC-coupled secondary antibody (green signal), PSV autofluorescence is shown in blue. Labeling was performed in germinated seeds. Scale bars are 10 μm. Insets of (C) and (D) show enlarged views of the green fluorescent signal of the periphery of the cavity by the Syp21 and mRab antibody, respectively (asterisks).

Fig. 5 Neutral Red labeling of living seeds. Neutral Red internalization (A) shows accumulation of this acidic marker inside the cavity (asterisk) of PSVs revealed by DIC (B) in seeds germinated for 24 h. Scale bar is 10 μm.
lytic vacuole via the PVC (Bolte et al. 2004). Both markers show punctate cytosolic labeling patterns in dry and stratified seeds (data not shown). In germinating seeds, we observed a massive accumulation of these punctate structures labeled by Sypr21 as well as to a lesser extent mRab lining in a pearl chain-like pattern at the periphery of the lytic cavity (Fig. 4C, D). We also observe that the pearl chain is connected to the outside of the cavity inside the PSV (Fig. 4C, inset). These results indicate a close vicinity of the newly formed lytic cavity with PVCs that might deliver the necessary molecular equipment for this vacuole upon germination.

Discussion

We have investigated the features and the dynamics of the vacuoles during the early stages of Arabidopsis seed germination. We detected structures labeled by TIP1;1 and AtNRAMP4 embedded in the PSV of dry seeds, which are no longer visible after stratification. Subsequently, after the seeds were germinated in the light for 1 d, cavities of heterogeneous sizes labeled by TIP1;1, V-PPase, V-ATPase but not by AtNRAMP4 become visible inside the PSV. The results obtained in this study uncover a rapid and dramatic reorganization of the vacuole during the early stages of Arabidopsis seed germination.

Distinct compartments with features of PSV or lytic vacuole coexist in cells of germinating seeds

We detected PSV and lytic vacuole markers at all three investigated stages of early germination. This is distinct from several published studies, where only α-TIP, but not γ-TIP was present in dry, stratified and 24 h germinated seeds: it has been shown by western blot that γ-TIP protein could not be detected earlier than 48 h after germination in Arabidopsis, pumpkin and mung bean seedlings (Höfte et al. 1992, Maeshima et al. 1994, Hunter et al. 2007, Wang et al. 2007), whereas α-TIP is abundant in dry seeds and during early germination. Poxleiner and collaborators could detect γ-TIP expression in Arabidopsis in stratified and germinated seeds but not in dry seeds (Poxleitner et al. 2006). Likewise, V-PPase and V-ATPase have been detected by western blot only 2 d after germination in pumpkin (Maeshima et al. 1994). In agreement with the lack of immunodetection of γ-TIP by western blot, studies using fluorescent fusion protein with isoforms of TIP in A. thaliana failed to visualize this isoform in dry seeds and during the early stages of germination (Hunter et al. 2007, Gattolin et al. 2009).

Nevertheless, the detection of γ-TIP, V-PPase and V-ATPase in seeds by immunofluorescence labeling has been reported by several groups. In immunofluorescence experiments, γ-TIP and V-PPase have been detected in dry seeds in tomato, snapdragon and tobacco (Jiang et al. 2001) and γ-TIP has been observed in Brassica and Arabidopsis (Gillespie et al. 2005). The γ-TIP labeling pattern reported by Gillespie et al. (2005) in dry seeds does not match the pattern observed in our study. Although both studies agree that γ-TIP labels structures embedded inside the PSV of mature Arabidopsis seeds, Gillespie and collaborators visualized a network distinct from the punctate structures that we observed (Gillespie et al. 2005). This discrepancy may be due to differences between cell types within the embryo; we focused on embryo axis cells whereas Gillespie et al. (2005) present an image of cotyledon cells. Alternatively, the divergent observation may be due to the different fixative procedures used in the two studies. The detection of proteins such as γ-TIP, V-PPase and V-ATPase in immunolabeling experiments in contrast to the absence of labeling in western blot experiments or using fluorescent fusion proteins is most likely due to differences in the sensitivity of the detection methods. Our findings using TIP1;1::GFP-expressing plants support this interpretation: although no GFP fluorescence is detectable in these plants during the early steps of germination either (Beeco et al. 2009), enhancing the signal by using anti-GFP antibodies coupled to FITC allows the detection and localization of the TIP1;1::GFP protein. Amplification of GFP signals by immunochemical staining using anti-GFP antibodies has already been useful in enhancing Golgi labeling in dendritic spines (Tomoka and Rockland 2006). In addition, in our study, immunolabeling of TIP1;1::GFP has been useful to increase the specificity of detection by enabling the visualization of a single TIP1 isoform: TIP1;1::GFP was found only on punctate structures in stratified seeds whereas γ-TIP antibody labeled both punctate structures and the tonoplast around the PSV, indicating that this antibody recognizes several TIP1 isoforms. Using the TIP1;1 promoter: TIP1;1::GFP-transformed plants, the detection threshold and labeling specificity were thus largely enhanced while dealing with endogenous expression levels of TIP1;1.

Distinct lytic compartments within the PSV in dry seeds and germinated seeds

Our results identified lytic compartments embedded in the PSV of dry and germinating seeds. They raise the question of the identity of these lytic compartments. Jiang et al. (2001) proposed that the globoid cavity in dry seeds of tobacco is surrounded by a membrane and they detected γ-TIP and V-PPase on this membrane in dry seeds. In addition, they found that γ-TIP and V-PPase are associated with purified globoids. The presence of γ-TIP on the structures embedded in the PSV in dry seeds suggests that they correspond to globoids. Furthermore, we observed V-PPase on these structures (Supplementary Fig. S4) co-localizing with TIP1;1::GFP. The presence of NRAMP4 is also in agreement with this interpretation as NRAMP4 metal transporter has been proposed to remodelize iron localized in the globoids of dry seeds (Lanquar et al. 2005). Our results are consistent with the notion that the PSV-embedded structures in dry seeds are globoids. However, there is a discrepancy in size between the 0.2–1 μm globoids observed in Arabidopsis seeds using transmission electron microscopy (TEM) (Lanquar et al. 2005, Poxleitner et al. 2006, Ebine et al. 2008, Shimada et al. 2008, Van Son et al. 2009, Takahashi et al. 2010) and the 2–4 μm structures we detect.
using γ-TIP, NRAMP4 or V-PPase antibodies. One explanation for this discrepancy could be that we specifically imaged cells of the embryo axis whereas the TEM images were taken from other parts of the embryo and the morphology of the PSV may differ between different cells within the embryo. Another explanation lies in the very different fixation and embedding methods used for TEM and immunofluorescence experiments, which could modify the aspect of globoids.

Several lines of evidence indicate that the lytic structures embedded in the PSV of dry seeds and the cavities in the PSV of germinating seeds are distinct: (i) NRAMP4 labels the lytic compartment in dry seeds but not in germinating seeds (Fig. 2); (ii) no compartment embedded in the PSV is labeled by either γ-TIP or AtNRAMP4 in stratified seeds, and (Fig. 2, 3); (iii) the cavities formed during germination are more heterogeneous in size and not labeled by AtNRAMP4 (Fig. 1). They have the hallmarks of the future central lytic vacuole of mature root cells: the presence of proton pumping V-ATPase and V-PPase (Fig. 4), and an acidic lumen (Fig. 5). However, their appearance might be delayed in cotyledons, which would explain the differences in the timing of vacuole dynamics between this study and the results we reported earlier (Lanquar et al. 2005). It has been shown in vetch that the temporal pattern of globulin storage protein mobilization is different in cotyledons in comparison with the embryonic axis: during the first 2 d after stratification, the embryonic axis is responsible for amino acid supply. Globulins are mobilized in cotyledons only after depletion of the embryo stores (Tiedemann et al. 2000). Accordingly, we observed a slight delay in cavity formation between the embryonic axis and the cotyledons in differential interference contrast (data not shown).

A possible scenario for vacuole remodelling during germination

Based on our results, we propose the following scenario for vacuole dynamics in germinating Arabidopsis seeds: first the fast mobilization of globoid mineral stores would occur during seed stratification and lead to subsequent disappearance of the globoids. Secondly, during germination a novel lytic compartment would be formed. The variability in the expansion rate of this newly formed compartment in different cells during germination would account for the heterogeneous size range. We propose that this new lytic compartment is involved in building the tunescence necessary for the emergence of the radicle and/or in processing and mobilization of the proteins stored in the PSV. What are the origins and how does this lytic vacuole form? We find that the prevacuolar markers Syt21 and mRab1 line up in close vicinity to this compartment. The small punctate cytosolic vesicles labeled with γ-TIP in close vicinity to the PSV in stratified seeds may represent the precursors of the cavity observed in germinating seeds. During germination, these precursors might be fueled by PVCs to build up larger structures that would push into the PSV. In a second step, this vacuole might then be engulfed into the PSV. At later time points, we speculate that the newly formed lytic compartment might fuse with the tonoplast of the PSV and release its lytic compounds into the PSV. A similar mechanism has been proposed recently for γ-TIP predomains in Arabidopsis (Beebo et al. 2009). Physiologically this mechanism would allow a rather rapid alteration of intravacuolar pH and thus provide an optimal environment to facilitate digestion of proteins stored inside the PSV. Very recently, Zheng and Staehelin (2011) proposed different mechanisms for the transformation of PSV into lytic vacuole in the root tip cell of germinating tobacco seeds. It is difficult to compare their observations with those reported in this study because they are based on a different technique (TEM) and were made on a different cell type in a different species.

In future studies, it would be important to follow the development and fate of the lytic compartment in a more detailed analysis and with better resolution in terms of space and time. This could be done using live imaging of fluorescent protein fusions, a technically challenging approach because of high levels of autofluorescence in seeds and low expression levels of some marker proteins. Further studies should also aim at defining the membrane organization and relationships between the different types of vacuole in Arabidopsis germinating seeds using higher resolution techniques such as TEM.

Material and Methods

Plant material and growth conditions

Seeds from AtTIP1;1 KO mutants expressing TIP1;1 under the control of its own promoter were obtained as described in Beebo et al. (2009). Arabidopsis Col-0 and AtTIP1;1 seedlings were germinated in liquid culture containing 2.5 mM H3PO4, 5 mM KNO3, 2 mM MgSO4, 1 mM Ca(NO3)2, Murashige and Skoog microelements, 1% sucrose, 1 mM MES adjusted with KOH to pH 6.1 and 50 μM FeEDTA (ABI5 medium) for 24 h at 21°C in the light after 2 d of stratification at 4°C in the dark. ’Stratified seeds’ refers to seeds incubated for 2 d in liquid medium in the dark at 4°C; ’germinated seeds’ refers to stratified seeds transferred for 24 h in the light at 21°C with slow orbital shaking (20 rpm).

Immunofluorescence

Immunostaining procedures were performed on tissue sections of dry, stratified and germinated A. thaliana seedlings embedded in polyethylene glycol as previously described (Boutté et al. 2006).

Primary antibodies were rabbit polyclonal antibody AtNRAMP4 1:200 (Lanquar et al. 2005), the lytic vacuole marker rabbit polyclonal antibody γ-TIP 1:200 (Jauh et al. 1999), the PSV-marker rabbit polyclonal antibody α-TIP 1:200 (Jauh et al. 1999), vacuolar H+-ATPase subunit A rabbit polyclonal antibody 1:500 (Zhigang et al. 1996), vacuolar pyrophosphatase rabbit polyclonal antibody 1:100 (Sarafian et al. 1992), anti-GFP mouse clone JL-8 (BD Biosciences) 1:100, the

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prevacuolar markers rabbit polyclonal antibody m-Rabmc 1:100 (Bolte et al. 2004) and Syp21 1:100 (da Silva Conceicao et al. 1997).

Single labeling was performed as previously described (Boutté et al. 2006) with anti-rabbit IgG coupled to FITC diluted 1:60 (Sigma). Single and double labeling of cells was performed with anti-rabbit and anti-mouse primary antibodies. Supplementary Figs 1, 2 show representative immunolabeling control experiments. For double labeling using two polyclonal antibodies, first primary antibodies were incubated at 4°C overnight followed by washing in PBST [PBS with 0.05% (v/v) Tween 20]. Cy3-conjugated Fab fragments were then added and incubated at room temperature for 2 h prior to a second wash, followed by postfixation and washing before the addition of the second primary antibodies. The Cy3-conjugated Fab fragment secondary antibodies (Intercam) were used at a dilution of 1:20, while FITC-conjugated secondary antibodies (Sigma) were used at a dilution of 1:60. Controls included labeling without the use of the second primary antibodies.

Neutral Red internalization

After removal of their seed coat, germinating Arabidopsis seedlings were incubated with 1.225 mM Neutral Red (stock 35 mM in H2O) for 1 h at room temperature (Fricker et al. 2001).

Confocal microscopy image analysis

Images were collected using a 63× oil immersion objective, NA 1.40 with a Leica laser-scanning confocal microscope TCS SP2 (Leica Microsystems, Heidelberg, Germany). Fluorochromes were detected sequentially using laser lines 405, 488 and 543 nm. The images were coded blue (autofluorescence), green (FITC) and red (Cy3). Images were processed using Image J (W.S. Rasband 1997–2009) and Adobe Photoshop (Adobe Systems). Fluorescence intensity profiles were carried out as described previously (Bolte and Cordelières 2006).

Supplementary data

Supplementary data are available at PCP Online.

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