Architectural remodeling of the tonoplast during fluid-phase endocytosis

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During fluid phase endocytosis (FPE) in plant storage cells, the vacuole receives a considerable amount of membrane and fluid contents. If allowed to accumulate over a period of time, the enlarging tonoplast and increase in fluids would invariably disrupt the structural equilibrium of the mature cells. Therefore, a membrane retrieval process must exist that will guarantee membrane homeostasis in light of tonoplast expansion by membrane addition during FPE. We examined the morphological changes to the vacuolar structure during endocytosis in red beet hypocotyl tissue using scanning laser confocal microscopy and immunohistochemistry. The heavily pigmented storage vacuole allowed us to visualize all architectural transformations during treatment. When red beet tissue was incubated in 200 mM sucrose, a portion of the sucrose accumulated entered the cell by means of FPE. The accumulation process was accompanied by the development of vacuole-derived vesicles which transiently counterbalanced the addition of surplus endocytic membrane during rapid rates of endocytosis. Topographic fluorescent confocal micrographs showed an ensuing reduction in the size of the vacuole-derived vesicles and further suggest their reincorporation into the vacuole to maintain vacuolar unity and solute concentration.

Introduction

Plant storage cells accumulate a portion of their photoassimilates in the vacuole via fluid phase endocytosis (FPE),1,6 a process characterized by the nonspecific bulk uptake of extracellular solutes in plasma membrane-bound vesicles.7 The portion of assimilates taken up by FPE varies according to the apoplastic solute concentration,8 the remaining difference being transported by membrane-bound carriers. Although firmly established that all types of endocytic systems (i.e., clathrin-mediated, caveolin-mediated, lipid raft-dependent, flotillin-dependent and macropinocytosis)9 carry a fluid-phase component,10,11 FPE is distinguished by their relatively larger vesicle size11,12 and cargo capacity. Solutes trapped by FPE eventually reach the vacuole;13,14 however, some systematic cargo distribution within the endosome has been observed using a combination of soluble membrane-impermeable endocytic markers5,33 and fluorescent nano-particles,16

In plants, FPE has been documented primarily for storage cells.3,6,8,17,19 During accumulation of photoassimulates at low external concentrations, membrane-bound solute transporters support the cellular energy and metabolic demands, in addition to transporting surplus solutes to the vacuole.20-22 This constitutes the hyperbolic, high-affinity phase of the characteristic curvilinear photoassimilate uptake mechanism.23 At high extracellular sucrose, mannitol or glucose concentrations, FPE is triggered and functions largely to carry surplus photoassimilates into the vacuole bypassing the cytosol,13,15 constituting the linear phase of the uptake curve.23 The simultaneous action of these multiple transport mechanisms was demonstrated using fluorescent deoxy-glucose, evacuated cytoplasts and inhibitors of both transport systems24 and their coordinated action detailed by Pozueta-Romero et al.8 Similar conclusions were obtained by Bandmann and Homann3 with tobacco BY-2 cells. The results provided evidence that FPE and membrane transporters can work in parallel and are under strict control of the external photoassimilate supply. A number of studies have revealed the formation of endocytic vesicles of different sizes ranging from 0.5–6 μm,6,26 a size more in accordance with macropinocytosis.26 However, membrane capacitance measurements by Bandmann and Homann3 and Gall et al.27 suggested an endocytic vesicle diameter of 80–220 nm. Whether or not the larger vesicles carrying endocytic markers observed during FPE result from the
variations in the lysosome volume during macropinocytosis in macrophages, Swanson suggested a system of retrograde vesicles of much reduced in size compared with the endocytic vesicles, a way to minimize solute loss while disproportionally removing excessive membrane surface area. If the diameter of the efflux vesicles were considerably smaller than that of the macropinosome (endocytic vesicle) the cell could balance incoming and outgoing membrane, and at the same time, restrict the retro-efflux of fluid. Nevertheless, regardless of the size of the exocytic vesicles, some portion of the vacuolar fluids would be returned to the exterior, creating an ancillary futile cycle. The remaining water and other permeable solutes would cross the lysosome membrane leaving membrane impermeable solutes to concentrate in the lysosome. Such a system could explain how solutes are concentrated in the storage lysosome/vacuole thereby maintaining the membrane surface area without significant loss of trapped solutes.31 In support of this proposed system, clathrin fusion of smaller vesicles prior to merging with the vacuole has not been determined.

During the vesicle delivery process, the vacuole receives a considerable amount of membrane and fluid content which, if allowed to accumulate over a period of time, would invariably disrupt the structural equilibrium of the mature cells. Furthermore, given the disproportional membrane surface-area/volume added by endocytic vesicles, membrane influx in the absence of a compensatory efflux process would result in increased dilution of the vacuolar contents. Therefore a retrieval process must exist that would guarantee membrane homeostasis in light of tonoplast expansion by membrane addition during FPE. Despite the volumes of literature on endocytosis in both animal and plant cells, the structural integrity of the tonoplast/lysosome during endocytosis remains unexplained, largely overlooked by the difficulty in visualizing, measuring and quantifying such changes. In an attempt to explain the lack of observed variations in the lysosome volume during macropinocytosis in macrophages, Swanson suggested a system of retrograde vesicles of much reduced in size compared with the endocytic vesicles, a way to minimize solute loss while disproportionally removing excessive membrane surface area. If the diameter of the efflux vesicles were considerably smaller than that of the macropinosome (endocytic vesicle) the cell could balance incoming and outgoing membrane, and at the same time, restrict the retro-efflux of fluid. Nevertheless, regardless of the size of the exocytic vesicles, some portion of the vacuolar fluids would be returned to the exterior, creating an ancillary futile cycle. The remaining water and other permeable solutes would cross the lysosome membrane leaving membrane impermeable solutes to concentrate in the lysosome. Such a system could explain how solutes are concentrated in the storage lysosome/vacuole thereby maintaining the membrane surface area without significant loss of trapped solutes. In support of this proposed system, clathrin fusion of smaller vesicles prior to merging with the vacuole has not been determined.
coated retrieval vesicles have been observed at the surface of lysosomes, whereas retrograde membrane traffic systems from the vacuole have been identified as part of the membrane recycling process in *Saccharomyces cerevisiae*, in tobacco BY-2 cells and from the pre-vacuolar compartment in Arabidopsis.

Throughout our investigations into FPE in plant cells with a variety of membrane-impermeable probes, we consistently observed their eventual deposition in the vacuole. For such mature cells to endocytose constitutively, a stable tonoplast surface area/volume ratio must be preserved by an unknown accompanying process of membrane turnover. Based on reports of retrograde vesicle budding, the exceptional plasticity of the tonoplast and our unpublished observations, we hypothesize that the existence of one or several cellular, morphological and/or metabolic events that will assist in vacuolar stability during endocytosis. As part of our continued investigation on FPE in plant cells, we examined the morphological changes to the vacuolar structure during rapid rates of endocytosis. To follow these essential modifications, we used red beet hypocotyl tissue. The heavily pigmented vacuole of the storage parenchyma cells allowed us to microscopically visualize all architectural transformations during treatment and distinguish these from any possible plasma membrane-derived osmocytotic structures as those reported by Goodwin et al. and Oparka et al. The present study revealed that, under conditions of rapid FPE proliferation, the cell transiently counterbalances the addition of surplus membrane by vacuole fragmentation with a system of vacuole-derived vesicles. Topographic fluorescent confocal micrographs showed an ensuing reduction in the size of the vacuole-derived vesicles and further suggested their reincorporation into the vacuole.

### Results

**Endocytic sucrose uptake.** Protoplasts prepared from red beet storage tissue after incubation in 200 mM sucrose and the blue-fluorescent membrane-impermeable endocytic marker Alexa-350 for 24 h exhibited a pronounced blue fluorescence in the vacuole (Fig. 1A and C). The blue fluorescence was not due to autofluorescence by the vacuolar pigment betacyanin. Although betacyanin has a wide fluorescent spectrum (560–750 nm) it does not overlap with that of Alexa-350 (400–550 nm). Furthermore, and most notably, protoplasts prepared from control tissue incubated in Alexa-350 and betaine instead of sucrose showed no fluorescence (Fig. 1D). Sucrose induced FPE has been reported for other tissues including turnip storage parenchyma, citrus juice cells and sycamore cultured cells. Consistent with the participation of FPE in the process of sucrose uptake, determinations of membrane flux indicated a significant movement of plasma membrane elements to the tonoplast. A 20% increase in vanadate-sensitive ATPase activity (plasma membrane H+-ATPase marker) was measured in purified tonoplast fractions from tissue incubated in sucrose when compared with control samples incubated in equal concentrations of betaine (Table 1). The above observations are in agreement with other storage tissues and collectively indicate that transport of Alexa-350 to the vacuole (Fig. 1) was mediated in part by sucrose-induced FPE.

**Vacuole restructuring during endocytosis.** During dormant or resting stage, red beet hypocotyl storage cells contained predominantly one large central vacuole (Fig. 2A and B). However, after tissue incubation in 200 mM sucrose for 24 h, a series of vacuole-derived organelles become evident (Fig. 2C and D), a condition scarcely observed when betaine was used as osmotic control. These vesicles are unquestionably of vacuolar origin given their red betacyanin content (Fig. 2C and D). The vacuole-derived vesicles not only varied in size and number between cells from different beet hypocotyl preparations, but also between cells from the same hypocotyl. Lack of synchrony is not unusual since not all cells participate equally in the endocytic process.

The absence of vacuole-derived vesicles in protoplasts obtained from control tissue cultured in betaine is in clear contrast to the prominent tonoplast vesiculation observed by Diekmann et al. using guard cells protoplasts subjected to osmotic excursions. However, it is noteworthy that guard cells are highly differentiated cells primarily specialized to undergo rapid volume adjustments based on vacuole fragmentation and reformation in response to osmotic changes as opposed to sucrose accumulating beet storage parenchyma cells.

A Z-stack 3D scanning laser confocal analysis revealed the abundance of vesicles and their large variation in size (Fig. 3A and B). Whether the larger vacuole-derived vesicles resulted from the fusion of smaller ones cannot be determined from the data generated by these static images. Nevertheless, merger of vesicles would not offer the cell any organizational advantage during photoassimilate accumulation since fusion of two spherical compartments would generate a larger surface area/volume structure resulting in solute dilution.

Fluorescence comparison of vacuole and vacuole-derived vesicles revealed comparable fluorescence intensity (Fig. 4). Since signal strength is a function of the concentration of fluorescent molecules, the concentration of betacyanin (and hence all solutes) within the vacuole-derived vesicles must, therefore, be reasonably similar in all compartments (Fig. 4B). This apparent

### Table 1. Changes of plasma membrane marker vanadate-sensitive ATPase activity in red beet cells incubated in sucrose or betaine for 24 h

| Time 0 | Betaine | Sucrose | % increase |
|--------|---------|---------|------------|
| 0.17 ± 0.03 | 0.23 ± 0.01 | 0.31 ± 0.02 | 20% |

*One unit defined as 1 nkat. Purified tonoplast fractions were isolated from fresh tissue and from tissue incubated in sucrose or betaine for 24 h. Vanadate-sensitive ATPase activity was measured in the presence and absence of vanadate.*

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uniformity in fluorescence remained despite some expected pigment dilution generated by incoming endocytic fluids.

**Apparent volume contraction in vacuole-derived vesicles.** The vacuole-derived vesicles formed during incubation in sucrose underwent visible changes when cells were further incubated for an additional 24 h in the absence of sucrose. At the end of the additional incubation period, there were no observable vesicles, or when present, their numbers were substantially reduced suggesting a re-fusion process with the large central vacuole. The likelihood that the pigmented vacuole-derived vesicles merged with the plasma membrane in an exocytic manner was disregarded since there was no betacyanin bleeding into the media during incubation. During a more rigorous examination, the enduring vesicles appeared darker in color under light microscopy, although not so apparent in photomicrographs (Fig. 5A and B). However, the perceived increase in color intensity was confirmed by a landscape view of a topographic fluorescence analysis (Fig. 5C). The vesicle fluorescence peaks were an average 20–30% taller than those emanating from the vacuole indicating higher betacyanin concentration.

In a distinct type of image analysis performed on Z-stacks obtained from separate cells, topographic surface reconstruction of maximum fluorescence intensity analysis of protoplasts only revealed the outlining tonoplast (Fig. 6A and B). At this setting, fluorescence of the central vacuole was eliminated whereas the vacuole-derived vesicle(s) remained intensely fluorescent indicating that the concentration of betacyanin (and thus all membrane impermeable solutes) within the vacuole-derived vesicles was considerably higher than in the large central vacuole (arrows). The increase in fluorescence intensity strongly suggests that vacuole-derived vesicles underwent a process of size reduction and their disappearance denotes their eventual fusion with the vacuole.

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**Figure 2.** Light Nomarski (A and C) and fluorescent laser scanning confocal micrographs (B and D) of protoplasts from red beet storage tissue. Protoplasts were prepared from hypocotyls at dormant state (A and B) and after 24 h incubation in 200 mM sucrose (C and D).
In eukaryotic cells, the dynamics of membrane maintenance requires a constant turnover of endosome-intrinsic constituents mediated by vesicle transport and the processes of exocytosis and endocytosis. In each of these processes, membrane vesicles composed of lipid bilayers and various membrane proteins are pinched off from one organelle and delivered to other subcellular or extracellular compartments that are undergoing expansion or reconstruction. During cell growth, much of this trafficking is directed outwards toward the plasma membrane and, in plants, toward the enlargement and assembling of the vacuole. However, in non-expanding cells, membrane degrading and transport pathways must be kept in balance to maintain the proper functioning size of cellular constituents. As part of internal trafficking between different organelles, several inter-endosomal retrograde systems have been hypothesized that undoubtedly contribute to membrane homeostasis.

In mature plant storage cells, sugar accumulation takes place in the confined space of a non-expanding vacuole. Aside from sugar transport by tonoplast-bound transporters, the partial delivery of sugars in cargo-filed endocytic vesicles to the vacuole imposes a significant structural and spatial constraint difficult to overcome by the reported clathrin-dependent vacuolar retrograde systems. Merging of the relatively large FPE vesicles with the vacuole generates a dual imbalance of membrane and vacuolar fluids. The data we present in this communication confirm the endocytic uptake of extracellular fluids induced by sucrose (Fig. 1) and reveal the transitional re-arrangement of the tonoplast/vacuole architecture during this process (Figs. 2–6). Vacuole fragmentation allows for the temporary adjustment of membrane surplus and, at the same time, assuring the eventual concentration of solutes. This conclusion is supported by three interrelated pieces of evidence. First, incorporation of the fluorescent endocytic marker Alexa-350 into the vacuole in the presence of sucrose indicated the involvement of FPE. Second, a substantial and consistent fragmentation of the vacuole was observed upon sucrose accumulation by FPE (Figs. 2–4). Third, the increase intensity of pigment fluorescence denoted volume shrinkage of the vacuole-derived vesicles (Figs. 5 and 6). Although speculative at this moment, disappearance of the vacuole-derived vesicles suggests their merging with the vacuole. A similar conclusion was reached for Saccharomyces at times of membrane expansion where vesicle trafficking and nuclear membrane deformations maintain nuclear/cell volume ratio. In plant cells, fragmentation of the vacuole is commonly observed in glandular cells of carnivorous plants during endocytosis. Another type of tonoplast deformation (termed “bulbs”) has been implicated in the rearrangement of the vacuole. Bulbs are tonoplast-delimited spherical protrusions of 1–3 μm and formed from cytoplasmic folds protruding into the vacuole. These are believed to serve as reservoir of tonoplast to prepare for quick volume changes.

The process of vacuole fragmentation described here is fundamentally similar in concept to that conjectured by Traub et al. and Swanson in that excess membrane from the vacuole/lysosome is removed in a retrograde manner. However, our findings differ in that the “retrograde” vesicles generated during FPE are unequally sized and are not recycled within the endosome as would be those using a clathrin-assisted vesicle system. Fundamental to the reduction in size of the vacuole-derived
vesicles (Figs. 5 and 6) is the participation of an additional, yet unknown, ancillary mechanism(s) involved in the removal of membrane components and volume regulation. Much of this permanent reduction can be performed by vacuolar lipases, which have been shown to be essential for membrane digestion during the macroautophagocytic process in yeast. In fact, mutation of cvt17, which encodes for a putative lipase in yeast, disrupted the proper turnover of endocytic membrane. In our experiments, it is possible that tonoplast digestion also took place in the large vacuole; however, since its volume was considerably larger than that of vacuole-derived vesicles, any reduction in size would have been proportionally smaller than that of vesicles. This disproportional reduction in size would allow the vacuole-derived vesicles to outshine the vacuole (Figs. 5 and 6).

Involvement of other proposed mechanisms that could conceivably participate in reducing or preventing expansion of the tonoplast during FPE are not supported by our data. For example, macrophagocytosis, the engulfing of protoplasm into the vacuole by tonoplast foldings, if operative, would have appeared as discolored areas within the vacuole, a situation never observed in our 3-D reconstructions of Z-stacks. The same absence of membrane inclusions makes microphagocytosis of vacuolar tubules an unlikely event. Finally, “kiss-and-run,” the fast release of contents by the endocytic vesicle into the targeted organelle and its immediate retrieval, can be disregarded by the observed increase presence of plasma membrane P-ATPase in vacuolar fractions (Table 1).

The tonoplast’s inherent elasticity is often overlooked as an essential factor in vacuolar maintenance. Capable of withstanding substantial deformations, the tonoplast can absorb extensive changes in surface area without the need for permanent removal. In fact this elasticity is likely the initial buffer mechanism during FPE before the formation of vacuole-derived vesicles.

Studies of membrane rearrangement during endocytosis have been precluded by the absence of an indisputable recycling cargo, a limitation overcome in this study by the use of red beet hypocotyl cells. These data indicate that tonoplast homeostasis during FPE is a coordinated event that may involve one or many processes. Here, we present evidence for the architectural remodeling of the tonoplast during rapid rates of FPE and suggest the participation of an axillary membrane recycling mechanism where vacuole-derived vesicles diminish their size before re-incorporation to the vacuole and maintaining vacuolar unity.

Materials and Methods

Plant material. Fresh red beet hypocotyls were purchased at a local grocery store and maintained refrigerated until use. Beets were brought to room temperature 24 h before experimentation. Tissue disks of approximately 0.5 mm in thickness were obtained from cylinders excised using a #5 cork borer. The disks were thoroughly rinsed in deionized water until bleeding stopped. After blotting dry, 1 g of tissue was incubated in a 25 mL Erlenmeyer flask containing 5 mL of a solution of 10 mM MES (pH 5.6), 200 mM sucrose, 1 mM CaCl₂, 1 mM MgCl₂ and 2 mM DTT, incubated for 24 h at 30°C while being agitated at 80 rpm. Treatment solutions were changed every 24 h as required by individual experiments. When indicated, inhibitors of endocytosis (10 μM Latrunculin-B) and
Protoplast preparation. Protoplasts were prepared from tissue incubated according to individual experiments by replacing the incubation medium with 10 mL of 100 mM MES (pH 5.6), 2% Cellulase from Trichoderma viride (Onozuka-RS; SERVA Cat no. 16420), 2% Pectinase from Aspergillus niger (CalBiochem Cat no. 515883), 1% Macerozyme R-10 from Rhizopus sp (SERVA cat no. 28302), 1% BSA, 125 mM CaCl$_2$, 125 mM KCl and incubated overnight in a rotary shaker at 30°C and 40 rpm. Prior to microscopic observation, the protoplasts were washed with a similar solution without hydrolyzing enzymes.

membrane-bound sucrose carriers (1 μM pCMBS) were added to pre-incubation and incubation solutions. The pre-incubation solution was similar to the incubation solution with 200 mM betaine added instead of sucrose.

At the end of the incubation time, tissue was rinsed three times with 5 mL of pre-incubation media for 10 min each. Samples were immediately frozen in 5 mL of 25 mM Tris/MES (pH 5.6) until further analysis. Sucrose was determined following the procedure of Van Handel$^{15}$ after thawing the tissue in a rotary shaker for 1 h at 40 rpm and 30°C.

Figure 5. Red beet protoplast isolated from tissue incubated 24 h in 200 mM sucrose followed by a 24 h period of sucrose starvation. (A and B) represent fluorescent and light laser scanning confocal micrographs, respectively. (C and D) are topographic analyses of (A and B), respectively. Fluorescent peaks on (C) correspond to the vacuole-derived vesicles in (A).
Tonoplast and plasma membrane vesicle isolation. Highly purified tonoplast and plasma membrane samples were isolated using a discontinuous sucrose gradient as described by Bennett et al.\textsuperscript{56} and Etxeberria and Gonzalez.\textsuperscript{57} The purities of the membrane fractions were corroborated measuring the total ATPase activities with and without vanadate and nitrate inhibitors. Individual membrane fractions were brought to 1.0 mL with storage buffer containing 250 mM sucrose, 10 mM MES pH 5.6 and 2 mM DTT and stored at −80°C.

**ATPase activity.** V-ATPase activity in tonoplast fractions was determined by coupling the production of ADP to the oxidation of NADH at 340 nm using a pyruvic kinase/lactate dehydrogenase system (PK-LDH). ATPase reaction solution contained 50 mM BTP/MES (pH 7.5), 4 mM dithiothreitol (DTT), 0.4 mg/mL bovine serum albumin (BSA), 250 mM sorbitol, 50 mM KCl, 10 mM gramicidin, 4 mM ATP, 4 mM MgSO\textsubscript{4}, 5 mL PK:LDH enzymes (Sigma P-0294), 0.25 mg/mL NADH, 1 mM phosphoenolpyruvate and approximately 100 mg vesicle protein in a total volume of 1.0 mL.\textsuperscript{58} ADP generated was calculated from the extinction coefficient of NAD (6.22) at 340 nm.

All reactions were carried with and without 50 mM vanadate at 30°C in a Shimadzu UV-160.

**Microscopy.** Light microscopy was performed using an Olympus BH-2 microscope equipped with a Canon Power Shot S3-15 digital camera connected to a Dell monitor. Fluorescent images were obtained using a Leica TCS-SL confocal laser scanning microscopy.

Given the wide range of fluorescence emission by the vacuolar pigment betacyanin, for experiments containing the red fluorescent membrane marker FM 4-64 FX, we used ex/em = 488/490-510. We captured FM 4-64 fluorescence at ex/em = 543/650-750. For all other experiments not containing FM 4-64, betacyanin was observed at ex/em = 543/590-650. Images were analyzed and created using Leica imaging system. To observe protoplast loaded with Alexa 350 we used a Zeiss Scope A-1 fluorescent microscope equipped with Zeiss Filter No. 49 (DAPI; Ex: G365, Em:BP445/50) and Zeiss Axio Cam ICc1.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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