

Plant Vacuoles

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INTRODUCTION

The vacuoles of plant cells are multifunctional organelles that are central to cellular strategies of plant development. They share some of their basic properties with the vacuoles of algae and yeast and the lysosomes of animal cells. They are lytic compartments, function as reservoirs for ions and metabolites, including pigments, and are crucial to processes of detoxification and general cell homeostasis. They are involved in cellular responses to environmental and biotic factors that provoke stress. In the vegetative organs of the plant, they act in combination with the cell wall to generate turgor, the driving force for hydraulic stiffness and growth. In seeds and specialized storage tissues, they serve as sites for storing reserve proteins and soluble carbohydrates. In this way, vacuoles serve physical and metabolic functions that are essential to plant life.

Plant cell vacuoles were discovered with the early microscope and, as indicated in the etymology of the word, originally defined as a cell space empty of cytoplasmic matter. Technical progress has variously altered the operating definition of the plant vacuole over time. Today, definitions continue to be colored by the tools and concepts brought to bear in any given study. Indeed, the combination of microscopy, biochemistry, genetics, and molecular biology is fundamental to research into the plant vacuole.

In this review, vacuoles are provisionally defined as the intracellular compartments that arise as a terminal product of the secretory pathway in plant cells. They are ontogenetically and functionally linked with other components of the vacuolar apparatus (i.e., vacuoles and those membranous bodies that are either committed to becoming vacuolar or have immediately completed a vacuolar function). Experimental evidence suggests that material within the vacuolar system in plants derives confluent from both an intracellular biosynthetic pathway and a coordinated endocytotic pathway. The biogenetic pathways include (1) sorting of proteins destined for the vacuole away from those to be delivered to the cell surface after transit through the early stages of the secretory pathway; (2) endocytosis of materials from the plasma membrane; (3) autophagy pathways for vacuole formation; and (4) direct cytoplasm-to-vacuole delivery. Ultimately, sorting and targeting mechanisms ensure that

specific proteins are faithfully assigned to conduct the vacuolar functions. The reader is referred to other contributions to this issue (i.e., Battey et al., 1999; Sanderfoot and Raikhel, 1999) and to previous reviews (Herman, 1994; Okita and Rogers, 1996; Bassham and Raikhel, 1997; Marty, 1997; Robinson and Hinz, 1997; Neuhaus and Rogers, 1998; Herman and Larkins, 1999) for detailed information on specific aspects of vacuole biology.

THE DIVERSITY OF VACUOLES

Plant cell vacuoles are widely diverse in form, size, content, and functional dynamics, and a single cell may contain more than one kind of vacuole. Although major morphological differences were recorded by the very first microscopists, it has been commonly assumed that all vacuoles have the same origin and belong to a common group. However, with improvements in cell fractionation and biochemical analyses as well as in the use of new molecular probes, it has become possible to characterize specialized vacuolar compartments in the cells from a variety of tissues (Hoh et al., 1995; Paris et al., 1996; Fleurat-Lessard et al., 1997; Swanson et al., 1998; Webb, 1999, in this issue).

In most cells from the vegetative tissues of the plant body, the central vacuole occupies much of the volume and is essential for much of the physiology of the organism. Among the many functions of this organelle are turgor maintenance, protoplasmic homeostasis, storage of metabolic products, sequestration of xenobiotics, and digestion of cytoplasmic constituents. In regard to the latter function, vacuoles are acidic and contain hydrolytic enzymes analogous to the lysosomal enzymes of animal cells. The membrane, or tonoplast, of such vacuoles contains the vegetative-specific aquaporin γ -TIP (for tonoplast intrinsic protein; Höfte et al., 1992; Marty-Mazars et al., 1995; Paris et al., 1996; Barrieu et al., 1998; see below). In some cell types, defense or signal compounds are stored in the vacuole, particularly within specialized cells located in strategically favorable tissues such as the leaf epidermis. As early as last century, it was observed that many pigments (e.g., anthocyanins) are localized in the vacuoles of epidermal cells from flowers, leaves, and stems. Recent findings suggest that the membranes of

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such specialized vacuoles contain specific ATP binding cassette (ABC) transporters (Rea et al., 1998).

In contrast, reserve tissues of seeds and fruit contain vacuoles specialized in the storage of proteins (Okita and Rogers, 1996; Müntz, 1998; see Herman and Larkins, 1999, in this issue). The membrane of the protein storage vacuoles (PSVs) contains the seed-specific aquaporin α -TIP (Höfte et al., 1992; Paris et al., 1996; Swanson et al., 1998; see below). Storage proteins are also synthesized and accumulated in specialized vegetative cells in response to wounding and to developmental switches (Maeshima et al., 1985; Sonnewald et al., 1989; Staswick, 1990; Herman, 1994; Jauh et al., 1998). Distinctively, the membrane of the vegetative storage vacuoles contains the aquaporin δ -TIP (Jauh et al., 1998; Neuhaus and Rogers, 1998). In the endosperm of cereal grains, proteins accumulate in endoplasmic reticulum (ER)-derived organelles of vacuole-like size (see below).

A few recent studies show that distinct vacuoles may simultaneously function in the same cell. Two separate vacuolar compartments, defined by α -TIP and γ -TIP, occur together in the root tip cells of barley and pea seedlings, mature tobacco plants, as well as in the plumule cells of pea seedlings (Paris et al., 1996). Barley lectin in root tip cells is found within α -TIP-positive vacuoles but not in γ -TIP-positive vacuoles, whereas the barley acid cysteine protease, aleurain, is specifically contained within γ -TIP-positive vacuoles but is absent from α -TIP-positive vacuoles. Thus, α -TIP defines a storage vacuole in which proteins are protected against degradative enzymes, whereas γ -TIP defines a separate, acidic, lytic vacuole. As cells develop large vacuoles, these two compartments appear to merge because the marker membrane antigens, α -TIP and γ -TIP, colocalize to the same membrane, at least in certain regions of the vacuolar compartments (Paris et al., 1996).

Two distinct vacuole types are similarly found in living protoplasts of barley aleurone (Swanson et al., 1998). In addition to PSVs, aleurone cells contain a second type of lytic organelle, designated as secondary vacuoles by the authors of this study. Although PSVs and secondary vacuoles are lytic organelles with acidic contents, it was suggested that the secondary vacuoles, which have many features typical of plant vacuoles, function as lysosomes and could be involved in the programmed death of aleurone cells (Swanson et al., 1998).

Another example of the versatility of vacuoles comes from investigations of the motor cells of the pulvini from *Mimosa pudica* (Fleurat-Lessard et al., 1997). The vacuole that occurs in the immature (nonreactive) motor cell is located near the nucleus, contains large amounts of tannins, and is believed to act as a Ca^{2+} store. The "aqueous" vacuole that is additionally found in mature motor cells does not contain tannins, is much larger than the tannin-containing vacuole, and occupies a central position in mature cells. The changes in cell volume that are responsible for pulvini-mediated leaf movement result from massive water fluxes mainly across the membrane of the larger, aqueous vacuole, on which the γ -TIP aquaporin and the vacuolar-type H^+ -translocating

ATPase (V-ATPase) are detected almost exclusively (for a review of membrane ATPases, see Sze et al., 1999, in this issue). Both vacuoles change shape to effect cell shrinkage. The tannin-containing vacuole forms interconnected tubules, whereas the aqueous vacuole develops membrane wrinkles. In any case, the tannin vacuole and the aqueous vacuole do not merge but rather coexist within the mature motor cell.

Additionally, because vacuoles are highly dynamic organelles, often capable of transforming in terms of both form and function, several "generations" of vacuole may be found within a given cell. In the cells of developing pea cotyledons, for instance, two categories of vacuole are reported: a declining, vegetative γ -TIP-associated vacuole; and a newly formed, α -TIP-associated storage vacuole (Hoh et al., 1995). Moreover, in suspension-cultured cells subjected to sucrose starvation, protein degradation is supported by numerous active autophagic vacuoles that are present together with the large, more mature central vacuole (Aubert et al., 1996; Moriyasu and Ohsumi, 1996). After completion of autophagic digestion, the small vacuoles are subsequently incorporated into the central vacuole. However, when intracellular digestion is inhibited, autophagic vacuoles containing undigested substrates remain in the cytoplasm as residual bodies, apart from the large central vacuole.

The diversity of function and form outlined in the above examples illustrates that the cytological definition of vacuoles is likely to cover several biochemically and physiologically distinct entities. Vacuoles, as dynamic organelles, can thus be viewed in the right perspective only if their dynamic nature itself is understood. In several instances, entities that may be variously defined according to different morphological, biochemical, and physical criteria may not necessarily correspond to distinct physiological units.

BIOGENESIS OF VEGETATIVE VACUOLES

Until recently, our knowledge of the biosynthesis and maintenance of vacuoles was based largely on morphological observations. Technological breakthroughs over the past few years have advanced our understanding of vacuolar biogenesis to a more detailed molecular level. Resident vacuolar proteins as well as proteins destined for degradation are delivered to the vacuole via the secretory pathway, which includes the biosynthetic, autophagic, and endocytotic transport routes that are presented in Figure 1. The basic mechanisms that organize these routes in eukaryotes are highly conserved across phyla (see Battey et al., 1999; Sanderfoot and Raikhel, 1999, in this issue).

Early Secretory Pathway

In plant cells, as in animal cells and yeast, anterograde transport of newly synthesized soluble as well as membrane

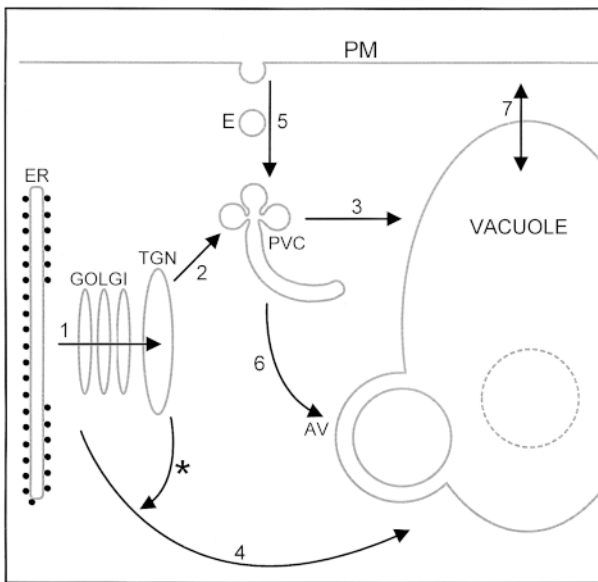


Figure 1. A Working Model for Transport Pathways in the Vacuolar Apparatus.

Seven basic pathways are used for the biogenesis, maintenance, and supplying of vacuoles. Pathway 1: entry and transport in the early secretory pathway (from ER to late Golgi compartments). Pathway 2: sorting of vacuolar proteins in the *trans*-Golgi network (TGN) to a pre/provacuolar compartment (PVC) via an early biosynthetic vacuolar pathway. Pathway 3: transport from PVC to vacuole via the late biosynthetic vacuolar pathway. Pathway 4: transport from early secretory steps (ER to Golgi complex; pathway 1) to the vacuole via an alternative route with possible material accretion from Golgi (indicated by the asterisk). Pathway 5: endocytic pathway from the cell surface to the vacuole via endosomes. Pathway 6: cytoplasm to vacuole through autophagy by degradative or biosynthetic pathways. Pathway 7: transport of ions and solutes across the tonoplast. AV, autophagic vacuole; E, early endosome; ER, endoplasmic reticulum; PVC, pre/provacuolar compartment; TGN, *trans*-Golgi network.

proteins through the vacuolar pathway begins at the ER. Most soluble proteins destined for the vacuole are synthesized as precursors with a transient N-terminal signal peptide by membrane-bound polysomes. The nascent precursor form is efficiently targeted to the ER lumen. After their cotranslational translocation across the ER membrane, the secretory proteins are folded and subjected to early post-translational modifications. ER-resident proteins, such as the luminal binding protein BiP (Denecke et al., 1991), assist newly synthesized polypeptides in acquiring their correct conformation. Proteins that fail to attain the correct three-dimensional structure are eventually degraded by a mechanism that does not involve the Golgi complex-mediated route to the vacuole. Alternatively, some proteins that are not properly

folded in the ER are delivered back to the cytosol by reverse translocation across the ER (Pedrazzini et al., 1997; Frigerio et al., 1998; see Vitale and Denecke, 1999, in this issue).

Secretory proteins that are inserted into or translocated across the ER membrane can contain sorting signals required for their targeting to and/or retention in almost any of the compartments along the secretory pathway. For some proteins, the target organelle is the ER itself, and these proteins are not transported further. All other proteins competent for transport along the secretory pathway are carried to the Golgi complex via a still elusive vesiculo-tubular intermediate compartment. Indeed, tubular continuities have been shown to form direct linkages between the ER and the Golgi complex. Consequently, tubular transport might occur in a direction tangential, rather than perpendicular, to the Golgi stacks, in a manner that differs, therefore, from that usually assumed to operate in animal and fungal cells.

The Golgi complex has a pivotal role in the secretory pathway. In plant cells, it consists of a set of dispersed units (dictyosomes) surrounded by a proteinaceous matrix. Like its counterpart in animal cells, each morphological Golgi unit in the plant cell includes a Golgi stack and a *trans*-Golgi network (TGN; Marty, 1978; Staehelin and Moore, 1995; Dupree and Sherrier, 1998). The Golgi stacks consist of three discrete groups of cisternae (*cis*, *medial*, and *trans*) that can be defined by their distinct morphologies and by their cytochemical and biochemical properties. Covalent and conformational modifications of newly synthesized secretory proteins, which begin in the ER, are continued in the Golgi complex and post-Golgi compartments. As they are being processed, vacuolar proteins transit through the early stages of the secretory pathway together with proteins that are destined to be exported into the extracellular medium or delivered to the plasma membrane.

Late Secretory Pathway—From TGN to Prevacuoles

The TGN is a major branch point in the secretory pathway and is the site of multiple sorting events that separate proteins destined for exocytotic egress from those progressing to the vacuole. The TGN varies in size according to the specific function of the cell. Under the hypothesis that biogenetic and trafficking processes are modulated in response to specific cell requirements, comprehensive morphological studies have been performed in actively vacuolating cells. The processes involved in the formation of vacuoles and their partitioning during mitosis, for example, are conveniently studied in the differentiating cells of the root meristem. Figure 2 shows the partitioning of mitotic provacuole clusters into daughter cells.

In cells in which new vacuoles are being formed, the TGN consists of a twisted, polygonal meshwork of smooth-surfaced anastomosing tubules extending from a central disk-shaped cisternal cavity facing the Golgi stack. Via lateral linkages, a single TGN might be shared by several Golgi

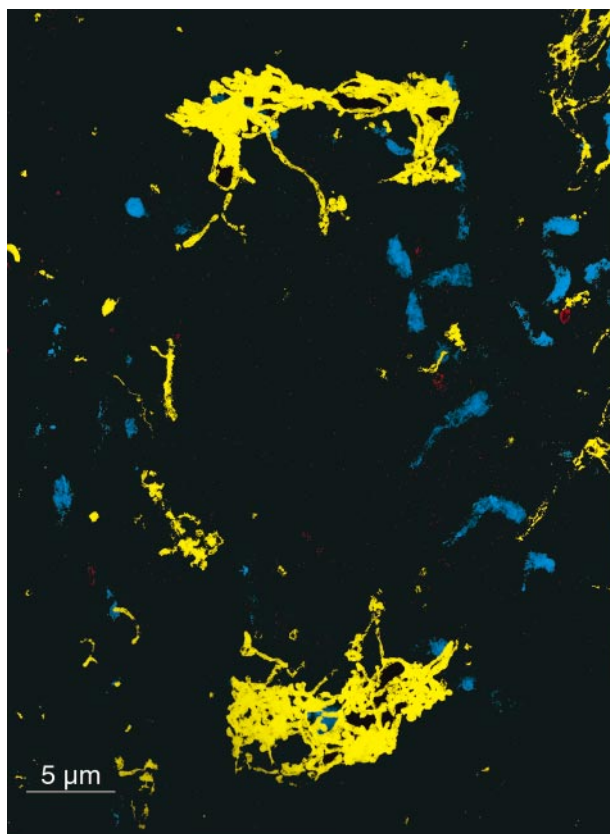


Figure 2. Partitioning of the Vacuolar Apparatus during Mitosis.

Distribution of mitotic provacuole clusters in vacuolating cells from the root meristem of horseradish. The vacuolar apparatus was selectively labeled by the zinc iodide–osmium reaction (see Marty, 1978). The specimen (3 μm thick) was examined without counterstain at 2.5 MV with a very high voltage (3 MV) electron microscope. Images were processed using Photoshop software (Adobe, San Jose, CA). Provacuoles are shown in yellow, Golgi complexes in red, and mitochondria and plastids in blue.

units. Clathrin-coated blebs and local swellings containing internal vesicles can be observed along the tubules, and numerous vesicles budding from the TGN mediate the transport of biomolecules to the vacuole.

The Prevacuolar Compartment

The TGN-derived vesicles on the vacuolar pathway form an intermediate compartment between the late *trans*-Golgi sorting site and the vacuole. These vesicles have been collectively referred to as *prevacuoles* because they act ontogenetically as the immediate *progenitors* of the vacuole. They also mediate transport between the ER/Golgi complex

and the vacuole and thus take functional *precedence* in the path to the vacuole. On account of this succession, they can be said to act as a physiological *prevacuolar* compartment (PVC) for cargo proteins en route to the vacuole (Marty, 1978, 1997).

Nascent provacuoles, budding from nodes of the TGN meshwork, have an average diameter (~ 100 nm) distinctly larger than the diameter of the TGN tubules (~ 15 nm). Rather quickly, the vesicular provacuoles extend into tubular provacuoles having roughly the same bore (100 nm) as the vesicles from which they derive. Their lumen is filled with vesicles that are presumably derived from microinvagination of their membranes (F. Marty, unpublished observations).

The extensive tubular provacuoles in vacuolating cells may be an enhanced version of the ubiquitous PVC described in mammalian cells and yeast (Piper et al., 1995). The membrane proliferation results from a dynamic effect that would occur either if membrane flow out of the provacuole were slowed down or if the membrane input from the TGN and/or the endocytotic tributary were increased. Furthermore, the provacuolar compartment might be a critical junction in post-Golgi trafficking at which the endocytotic and vacuolar biogenetic pathways converge.

Autophagy and Vacuolation

As revealed by three-dimensional high-voltage electron microscopy, the formation of autophagic vacuoles begins with a striking sequence of provacuole tubulation that proceeds to enclose discrete volumes of cytoplasmic material (Marty, 1978, 1997). Figure 3 represents this sequence of events, whereby tubular provacuoles produce digitate extensions that form cage-like traps so as to sequester portions of cytoplasm. Adjacent bars of the cage then fuse in a zipper-like fashion and, through transient palmar connections, build a continuous and tight cavity around the segregated portion of cytoplasm. Sections through these ball-shaped structures are recognized as early autophagosomes (i.e., a cytoplasmic area encircled by a narrow ring-like cavity bounded by inner and outer membranes).

Cytochemical studies show that the TGN, provacuoles, and autophagosomes are acidic and contain lysosomal acid hydrolases. The cytoplasm in the autophagosome is degraded after it has been totally closed off. It is speculated that the digestive enzymes are released from the surrounding cavity as the inner boundary membrane deteriorates. Upon completion of the digestive process, a typical vacuole is formed. The outer membrane, which remains impermeable to hydrolytic enzymes, confines digestive activities within the forming vacuole and becomes the tonoplast. Newly formed vacuoles can then fuse together to produce a few large vacuoles. Ultimately, facilitated transport of water through the tonoplast, mediated by γ -TIP aquaporins, results in rapid vacuole enlargement (Ludevid et al., 1992; Maurel, 1997).

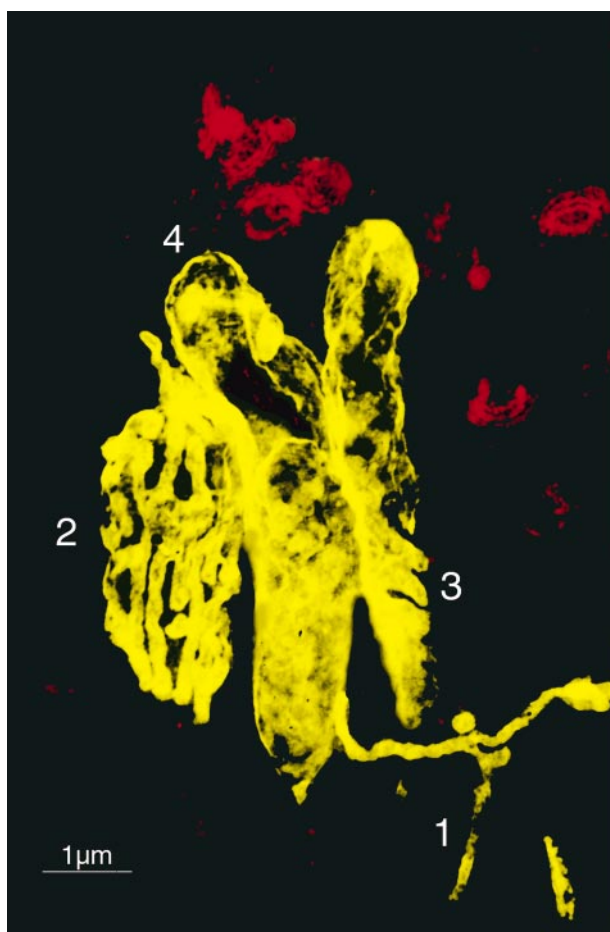


Figure 3. Autophagic Activity of Provacuoles.

Sequential stages of cytoplasmic confinement by provacuoles involved in cellular autophagy. Tubular provacuoles (1 and 2) form cage-like traps (2 to 4) enclosing portions of cytoplasm of a cell from the root meristem of *Euphorbia characias*. Adjacent bars of the cages then fuse to build a continuous and tight envelope (central structure [4]) around segregated portions of cytoplasm. Samples were processed as described in the legend to Figure 2.

Starvation-Induced Autophagy

In response to starvation, autophagy is reinitiated in cells that are already vacuolated. The autophagic pathway is activated, for example, after sucrose deprivation of suspension-cultured cells (Chen et al., 1994; Aubert et al., 1996; Moriyasu and Ohsumi, 1996). Portions of peripheral cytoplasm are first sequestered in double membrane-bounded envelopes (through the process described above) and then eventually digested. The small vacuoles, thus newly formed in the cytoplasm, are finally incorporated into the central vacuole. It has been shown that the induction of cellular autophagy is controlled by the supply of mitochondria with respiratory

substrate and not by the decrease in the concentration of sucrose and hexose phosphates (Aubert et al., 1996). Formation of autophagic vacuoles has been correlated with an increase in the rates of intracellular proteolysis (Moriyasu and Ohsumi, 1996) and a massive breakdown of membrane polar lipids (Aubert et al., 1996).

As a degradative pathway, autophagy plays a central role in protein and organelle turnover. It has been implicated in vacuolation and cell differentiation, and it is critical for survival during stress conditions such as nutrient deprivation. It can also be exploited for biosynthetic purposes as a cytoplasm-to-vacuole targeting pathway, as occurs in yeast, and with regard to supplying PSVs (see below).

VACUOLAR SORTING OF STORAGE PROTEINS

Specialized cells in seeds and vegetative organs accumulate proteins that function primarily as reserves of amino acids. The most common storage proteins are the globulins, which are found in embryos, and the prolamins, which are unique to cereal endosperms. Most storage proteins, including globulins and some prolamins, have been shown to be transported to vacuoles via the Golgi complex (Shotwell and Larkins, 1988; Chrispeels, 1991; see also Herman and Larkins, 1999, in this issue). However, studies on the assembly and transport of seed storage proteins in legumes and cereals have shown that reserve proteins can be sorted at diverse exit sites along the vacuolar pathway. As a result, proteins are stored in a variety of compartments specific to the plant species, tissue, stage of cell differentiation, and protein category.

Golgi-Dependent and Golgi-Independent Routes to PSVs

Pulse-labeling experiments, morphological and immunocytochemical studies, and biochemical analyses have provided compelling evidence for a Golgi-mediated route to the PSV in legumes and other dicots. Storage proteins are synthesized as precursors that are cotranslationally transferred into the lumen of the rough ER and transported via the Golgi apparatus into specialized vacuoles where proteolytic processing is usually needed to promote their stable storage.

Whereas protein storage deposits are seldom observed in the lumen of the rough ER at the early stage of the transport pathway, condensed storage proteins are commonly detected in smooth-surfaced vesicles, ~100 nm in diameter, in association with the *cis*-, medial-, or *trans*-cisternae of the Golgi complex (Hohl et al., 1996). Furthermore, three different types of vesicle are commonly found in close proximity to the Golgi area: vesicles that carry storage proteins, exocytotic vesicles containing cell wall polymers, and clathrin-coated vesicles (CCVs). The existence of two different exit

sites for vacuolar proteins at the Golgi complex and the utilization of "alternative" secretory vesicles suggest further variations to vacuole function (Gomez and Chrispeels, 1993). According to current views, vesicles containing storage proteins originate at the *cis*-Golgi cisternae, and proteins undergo maturation processes as they progress through the Golgi stacks up to the TGN, where they are sorted to the storage vacuoles (Robinson and Hinz, 1997). At the exit site, CCVs were found to bud off from the vesicles containing storage proteins. Subsequently, only the clathrin-free vesicles, but not the CCVs, are involved in the transport of soluble storage proteins to the vacuole.

A different pathway recently has been suggested in cells from maturing seeds of pumpkin and castor bean (Hara-Nishimura et al., 1998). In these cells, proglobulin and pro2S albumin were shown to be transferred from the rough ER to the PSV via large vesicles (200 to 400 nm in diameter). These large precursor-accumulating vesicles are distinct from the Golgi-derived vesicles but similar to the late protein bodies described in pea cotyledons (Robinson and Hinz, 1997). It was suggested that the core of storage proproteins contained in these large vesicles might derive directly from protein aggregates that are formed in the ER (Hara-Nishimura et al., 1998); they accumulate proprotein precursors and ER-resident proteins such as BiP but not mature products. In maturing pumpkin cotyledons, where the vast majority of storage proteins are not glycosylated, the precursor-accumulating vesicles bypass the Golgi apparatus such that their transport is not inhibited by the carboxylic ionophore monensin.

In contrast to pumpkin seeds, castor bean seeds contain storage glycoproteins with complex glycans. Their processing occurs in the Golgi complex. The Golgi-processed glycoproteins are subsequently incorporated into the ER-derived precursor-accumulating vesicles at the periphery of the core aggregates. Storage glycoproteins, together with other storage proteins, are ultimately transported by the mature vesicle as far as the PSV. However, the final steps of the transport pathway to the storage vacuole are still unknown. It was suggested that the incorporation of the precursors into PSVs could occur by membrane fusion or by autophagic engulfment of the vesicle into the vacuole.

Autophagy and PSVs

Developing legume cotyledons comprise a model system to study both the ontogenesis of the PSV and the intracellular transport of vacuolar reserve proteins (Chrispeels, 1991). In the parenchyma cells of maturing legume cotyledons, the very few large vegetative vacuoles become replaced by numerous PSVs. Ultrastructural studies indicate that the pre-existing vegetative vacuoles of immature parenchyma cells are trapped by a newly developing smooth tubulo-cisternal membrane system that already contains storage proteins (Craig, 1986; Hoh et al., 1995). However, the origin of this

new membrane system is not clearly understood. The trapped vegetative vacuoles disappear as the novel storage vacuoles gradually fill up with storage proteins (Hoh et al., 1995). During the process, the storage proteins aggregate as individual clumps against the tonoplast and cause it to protrude into the cytoplasm. By a budding process, the protruding protein masses, still surrounded by the tonoplast, become independent small storage vacuoles (membrane-bounded "protein bodies" [PBs]) dispersed in the cytoplasm.

At later stages of cotyledon maturation, the budding process stops, and the main original storage vacuole, which continues to accumulate reserve proteins, transforms into a distinct category of large storage vacuole. A third type of storage protein reservoir is formed in the cells at the middle to late stages of seed maturation, before storage protein synthesis ceases. Storage proteins accumulate in smooth-surfaced cisternae and channels with terminal dilations. These swellings may detach and become independent spherical bodies without cisternal connections.

Finally, in germinating legume seedlings, PSVs are replaced by a vegetative vacuole through yet another type of developmentally regulated sequestration and disposal of organelles. Local invaginations of the tonoplast and engulfment of cytoplasmic fragments, subsequently degraded in the PSV, have been described (Herman et al., 1981; Melroy and Herman, 1991).

Storage Proteins in Cereals

Cereal grains differ from legume seeds by accumulating the alcohol-soluble prolamins as storage proteins in endosperm cells (Shewry et al., 1995). Cereal prolamins, like legume globulins, are cotranslationally loaded into the lumen of the ER. In many cereals, including maize, rice, and sorghum, prolamins form dense, insoluble accretions, which are retained within the lumen of the ER and, as in the case of the legumes, termed PBs (Lending et al., 1988; Geli et al., 1994). In developing endosperm cells, PBs become enlarged as newly synthesized prolamins are acquired and assembled with the aid of protein disulfide isomerase and molecular chaperones such as BiP (Lending and Larkins, 1989; Boston et al., 1991; Li and Larkins, 1996).

Prolamins of other cereals, including wheat, barley, and oat, on the other hand, accumulate in vacuoles together with globulins (Shotwell and Larkins, 1988; Levanony et al., 1992). Globulins are transported along the anterograde pathway via the Golgi complex to the vacuolar compartment, whereas prolamins PBs are incorporated into the vacuole by an autophagic process (Levanony et al., 1992).

Several cytological observations have suggested that rather similar autophagic mechanisms might operate when transgenes encoding storage proteins from cereals are expressed in vegetative tobacco cells (Coleman et al., 1996; Bagga et al., 1997; Frigerio et al., 1998). The transgene

products form accretions in the ER, as in many storage cells in cereals, but the ER membrane-bound PBs are subsequently captured by an autophagic process and delivered to the vegetative vacuole, where they are eventually proteolytically degraded. Interestingly, somewhat similar steps could be detected during the transport of storage proteins to storage vacuoles by large precursor-accumulating vesicles in normally developing cells (Levanony et al., 1992; Hara-Nishimura et al., 1998; see above). These results suggest that the cellular machinery of autophagy can be used for delivering cytosolic proteins and early membrane-bound PBs to the vacuole, thus defining a biosynthetic cytoplasm-to-vacuole targeting pathway as occurs in yeast. The ontogeny of the compartments specialized in protein storage is thus diverse, and not all stores are (ontogenetically) homologous, although all belong to the vacuolar apparatus of plant cells. For a more detailed discussion of PSVs, see Herman and Larkins (1999), in this issue.

ENDOCYTOSIS

Endocytosis is defined as the uptake of extracellular and plasma membrane materials from the cell surface into the cell. Endocytosis has been characterized morphologically in plant cells in which both fluid-phase uptake and receptor-mediated internalizations have been visualized (reviewed in Low and Chandra, 1994; Marty, 1997; see also Battey et al., 1999, in this issue). Two distinct routes of internalization by clathrin-mediated endocytosis have been suggested to operate in plant cells: (1) from the plasma membrane to an endosomal compartment, including the partially coated reticulum, multivesicular bodies, TGN, and the PVC; and (2) from the plasma membrane to the PVC and the vacuoles (Low and Chandra, 1994). Novel intermediary structures arising from plasma membrane internalization have also been described as part of a compensatory recycling mechanism in actively secreting cells (Staehelein and Chapman, 1987). Rapid retrieval of plasma membrane to the cell interior, together with a fluid phase internalization of extracellular material, occurs in water-stressed cells (Steponkus, 1991; Oparka et al., 1993; Barrieu et al., 1999).

Morphological studies of vacuolating cells by electron microscopy suggest that the endocytotic and biosynthetic vacuolar pathways converge at the provacuolar compartment before nascent autophagic vacuoles are formed (F. Marty, unpublished observations). The convergence point(s) between these pathways in already vacuolated cells is unknown, but it seems reasonable to hypothesize that the juncture could be at the prevacuolar compartment. Endocytotic vesicles and endosomes belong to the vacuolar apparatus, but their direct contribution to the formation of the vacuole remains uncertain. Whereas the vesicle-mediated internalization of plasma membrane has been documented in plant cells, the routes involved need to be precisely

mapped by reliable tracers. A potential candidate is Tlg1p, a protein functionally homologous to the t-SNARE (see below) localized on a putative early endosome in yeast.

VACUOLAR SORTING SIGNALS

Vacuolar soluble proteins and membrane proteins alike travel through the early stages of the secretory pathway. Most probably, they are sorted away from proteins destined for delivery to the cell surface at the exit of the Golgi complex (see, e.g., Sanderfoot and Raikhel, 1999, in this issue). Soluble proteins therefore require a sorting signal to tag them for vacuolar delivery after their egress from the Golgi complex; indeed, in the absence of such informational tags, they are secreted to the extracellular space. Three types of vacuolar targeting signals have been described (Chrispeels and Raikhel, 1992). Some vacuolar proteins (e.g., sporamin and aleurain) contain an N-terminal propeptide (NTPP) as a targeting determinant; others (e.g., barley lectin, phaseolin, tobacco chitinase, and Brazil nut 2S albumin) contain a C-terminal propeptide (CTPP), whereas some vacuolar proteins (e.g., phytohemagglutinin and legumin) contain a targeting signal in an exposed region of the mature protein.

NTPP Signals

The targeting determinants characterized in NTPPs from the barley cysteine protease aleurain (Holwerda et al., 1992) and from sweet potato sporamin (Nakamura et al., 1993) contain a conserved Asn-Pro-Ile-Arg amino acid sequence. This motif in the NTPP is necessary and sufficient for the sorting of the sporamin precursor to the vacuole (Nakamura et al., 1993; Matsuoka et al., 1995). Sporamin is delivered to the sink vacuole in cells from the tuberous roots of the sweet potato (Maeshima et al., 1985), whereas aleurain is sorted to a lytic compartment distinct from the PSV (Paris et al., 1996).

CTPP Signals

By contrast to NTPP signals, a vacuolar sorting consensus sequence has not been identified in CTPP targeting domains. Nevertheless, the CTPP was shown to be necessary and sufficient for the targeting of barley lectin to the vacuole (Bednarek and Raikhel, 1991; Matsuoka et al., 1995). The N-linked glycan of the CTPP in barley lectin is not necessary for sorting, although it modulates the rate of processing of the propeptide. Hydrophobic residues in the CTPP are important for the targeting of barley lectin (Dombrowski et al., 1993). Similar mutagenesis analyses have been performed to characterize the targeting signal of tobacco chitinase (Neuhaus et al., 1994). CTPPs from vacuolar proteins differ in length, and it was recently shown that a short CTPP from

phaseolin contains information necessary for interactions with the vacuolar sorting machinery in a saturable manner (Frigerio et al., 1998).

The barley lectin, phaseolin, and Brazil nut 2S albumin accumulate in PSVs, whereas tobacco chitinase is delivered to vacuoles of vegetative cells. Results indicate that more than one sorting mechanism might exploit the CTPP targeting signal and that transport of CTPP-containing proteins from the Golgi complex to the vacuoles involves more than one pathway (Matsuoka et al., 1995; Frigerio et al., 1998). Both CTPP- and NTPP-mediated vacuolar delivery also involve alternative structures and mechanisms, although NTPP and CTPP were found to be functionally interchangeable in directing proteins to the vacuole (Matsuoka et al., 1995).

Internal Signals

Other plant vacuolar proteins are synthesized without a cleavable vacuolar-targeting signal. Studies on phytohemagglutinin (PHA) from *Phaseolus vulgaris* (Tague et al., 1990) and legumin from *Vicia faba* (Saalbach et al., 1991) have demonstrated targeting information in exposed regions of the mature proteins, which are deposited in the PSVs of the reserve parenchyma cells of cotyledons. Strikingly, soluble proteins, such as PHA, and proteinase inhibitors, which are usually vacuolar, occasionally have been detected in the extracellular matrix, suggesting that the vacuolar targeting signals might not be recognized in all cells (Kjemtrup et al., 1995). Moreover, recent work on suspension-cultured cells showed that some soluble, fully processed, vacuolar hydrolases can be excreted into the medium under hormonal control. The exocytotic pathway for these "vacuolar" proteins would lead from either the vacuole or the PVC situated downstream of the last processing step (Kunze et al., 1998).

Although short amino acid sequences of plant vacuolar proteins are sufficient to sort nonvacuolar proteins to the vacuole in yeast (Tague et al., 1990), plant proteins are sorted to the yeast vacuole by signals different from those recognized by plants, suggesting that the transport machinery is at least partially different between yeast and plants (Gal and Raikhel, 1994).

Vacuolar membrane and intravacuolar soluble proteins are targeted to vacuoles by different mechanisms. Pulse-chase experiments and pharmacological studies on protoplasts from transgenic tobacco plants suggest that soluble proteins such as PHA and integral membrane proteins such as α -TIP reach the same destination by traveling through different paths (Gomez and Chrispeels, 1993).

Signals in TIPs?

Transport pathways for integral membrane proteins of the tonoplast have been investigated (Höfte and Chrispeels, 1992; Jiang and Rogers, 1998). The vacuolar membrane

α -TIP and γ -TIP are polytopic integral membrane proteins, with six membrane-spanning domains and both N and C termini located in the cytoplasm. In an early analysis of the targeting information contained in α -TIP, it was found that a polypeptide segment comprising the sixth membrane-spanning domain and the adjacent C-terminal, cytoplasmic tail of α -TIP is sufficient to target a nonvacuolar reporter protein to the tonoplast. In addition, the C-terminal cytoplasmic tail was not found necessary for the targeting of α -TIP in the same stably transformed tobacco cells (Höfte and Chrispeels, 1992).

More recently, the trafficking of a chimeric integral membrane reporter protein was analyzed in tobacco protoplasts (Jiang and Rogers, 1998). It was found that the transmembrane domain of the plant vacuolar sorting receptor BP-80 (see below) directs the reporter protein via the Golgi complex to the prevacuolar compartment, and attaching the C-terminal cytoplasmic tail of γ -TIP did not alter this traffic. By contrast, attaching the C-terminal cytoplasmic tail of α -TIP prevented traffic of the reporter protein through the Golgi complex but caused it to be localized to vacuoles. It was thus concluded that there are two separate pathways to vacuoles for membrane proteins: a direct pathway followed by α -TIP from the ER to PSVs, and a separate pathway followed by γ -TIP via the Golgi complex and PVC to the vegetative lytic vacuole (Jiang and Rogers, 1998).

VACUOLAR SORTING RECEPTORS

Soluble vacuolar proteins are diverted away from the exocytotic pathway through a receptor-mediated process that leads to their delivery to the vacuole. Two independent approaches resulted in the identification of plant vacuolar sorting receptors (Kirsch et al., 1994; Ahmed et al., 1997).

It was initially hypothesized that the Asn-Pro-Ile-Arg motif conserved in the NTPP vacuole-targeting determinant of aleurain and sporamin, two unrelated proteins, was likely to be recognized by a sorting receptor (Kirsch et al., 1994). Indeed, a protein of 80 kD, called BP-80, has been affinity purified from a lysate of CCVs from pea. It possesses all the features expected of a vacuolar sorting receptor. It is a type I integral membrane protein that is localized in the Golgi complex and in small vacuolar structures. These vacuolar structures are distinct from both α -TIP and γ -TIP vacuoles but are possibly analogous to prevacuoles. Several homologs have been cloned, and the sequences appear to be highly conserved in monocotyledonous and dicotyledonous plants (Paris et al., 1997).

An alternative approach led to the identification of an Arabidopsis receptor-like protein called AtELP (for *Arabidopsis thaliana* epidermal growth factor-like protein). This second approach was based on the use of known functional motifs present in many of the receptor proteins involved in clathrin-dependent intracellular protein sorting in mammalian and

yeast cells (Ahmed et al., 1997). AtELP shares many common features with mammalian and yeast transmembrane cargo receptors. It is capable of *in vitro* interaction with the proteins of the TGN-specific AP-1 adaptor complex from mammals. It is located at the TGN, in CCVs, and on the PVC in the root cells of *Arabidopsis*. AtELP is highly homologous to BP-80, suggesting that it also may play a role in targeting proteins to the vacuole (Sanderfoot et al., 1998; see also Sanderfoot and Raikhel, 1999, in this issue). Mechanisms recognizing the C-terminal or internal vacuolar sorting signals of soluble proteins have not been elucidated, and the identification of receptor-mediated pathways for membrane proteins is still in debate (see, e.g., Vitale and Raikhel, 1999).

In addition to sorting receptors, other components of the vacuolar targeting machinery are being identified in plants. An interesting example is a V-ATPase activity associated with the Golgi complex, distinct from that of the tonoplast V-ATPase, and which is necessary for the efficient targeting of soluble proteins to the vacuole (Matsuoka et al., 1997; see Sze et al., 1999, in this issue).

TRAFFICKING STEPS AND SNARE COMPONENTS

Transport of soluble and membrane proteins in the secretory pathway is known to be mediated by the budding and fusion of transport vesicles (Rothman, 1994) and, in certain cell types or physiological situations, by cisternal progression and direct tubular linkages between different compartments (Pelham, 1998). As an early step in vesicular transport, budding involves coat proteins that assemble from the cytosol. CCVs, COPI and COPII-like vesicles, and "dense" vesicles have been described in plant cells (Robinson et al., 1998; see Sanderfoot and Raikhel, 1999, in this issue). Available data indicate that a considerable homology between coat proteins in plant, yeast, and animal cells exists, although we still know little of the molecular organization of transport vesicles in plants.

Docking and fusion steps are thought to be mainly regulated by integral membrane receptors, termed SNAREs (for soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) (see Sanderfoot and Raikhel, 1999, in this issue). According to the prevalent model, SNAREs on vesicles (*v*-SNAREs) interact with cognate SNAREs on the target membranes (*t*-SNAREs). The soluble proteins NSF (for *N*-ethylmaleimide-sensitive factor) and α -SNAP (for soluble NSF attachment protein) then bind the *v*-SNARE/*t*-SNARE complex, and a rearrangement triggered by ATP hydrolysis finally promotes membrane fusion. The diversity and specificity of vesicle transport routes correlate with the complexity of traffic effectors, which include Rab proteins, Rab-binding molecules, Ca^{2+} , and components of the cytoskeleton. Many lines of investigation suggest that the mechanisms of vesicular budding, docking, and fusion are conserved across species and subcellular compartments.

A growing number of proteins functionally homologous (orthologs) to the SNAREs characterized in yeast and mammalian cells is being identified in plant cells (Sanderfoot and Raikhel, 1999, in this issue). Initial results show that the sorting mechanism for soluble proteins to the plant vacuole agrees well with the SNARE model. The putative plant vacuolar receptor AtELP (see above) is able to recruit the adaptor complex 1 (AP-1) present at the TGN. As a consequence, the AtELP receptor appears to be included in TGN-derived CCVs. These vesicles carry the vacuolar cargo together with its receptor to the prevacuolar compartment, where the receptor (AtELP) and the prevacuole-specific *t*-SNARE (AtPEP12p; da Silva Conceição et al., 1997) are colocalized (Sanderfoot et al., 1998). The vacuolar *t*-SNARE AtVam3p is used downstream in the late vacuolar pathway. However, its function in homotypic (vacuole-vacuole) or heterotypic (prevacuole-vacuole) fusions or in autophagy is still being debated.

Compelling microscopic evidence is also suggestive of transient tubular continuities between compartments of the vacuolar pathway in particular cell types or physiological conditions. Indeed, tubular continuities between the ER and the Golgi complex, between cisternae from the same Golgi stack, between TGN units from adjacent Golgi stacks, between the TGN and the pre/provacuolar compartment, and between provacuoles and autophagic vacuoles have been described (see Marty, 1997). Such interconnections are consistent with an intracellular transport by cisternal progression and maturation. Vesicular and nonvesicular transport mechanisms, it should be stressed, are not mutually exclusive.

TONOPLAST FUNCTIONS

The vacuole plays an important role in the homeostasis of the plant cell. It is involved in the control of cell volume and cell turgor; the regulation of cytoplasmic ions and pH; the storage of amino acids, sugars, and CO_2 ; and the sequestration of toxic ions and xenobiotics. These activities are driven by specific proteins present in the tonoplast and indicated in Figure 4. These functions have been abundantly documented and reviewed (Sze et al., 1992; Rea and Poole, 1993; Barkla and Pantoja, 1996; Leigh, 1997; Maurel, 1997; Wink, 1997; Rea et al., 1998; see also Chrispeels et al., 1999; Sze et al., 1999, in this issue).

According to the chemiosmotic model for energy-dependent solute transport, the proton-motive force generated by either the V-ATPase or the H^+ -translocating inorganic pyrophosphatase (V-PPase) can be used to drive secondary solute transports. Movement of ions and water down their thermodynamic potentials is achieved by specific ion channels and water channels (aquaporins). The resulting ion, water, and metabolite fluxes across the vacuolar membrane are crucial to the diverse functions of the vacuole in plant cells, such as cell enlargement and plant growth, signal

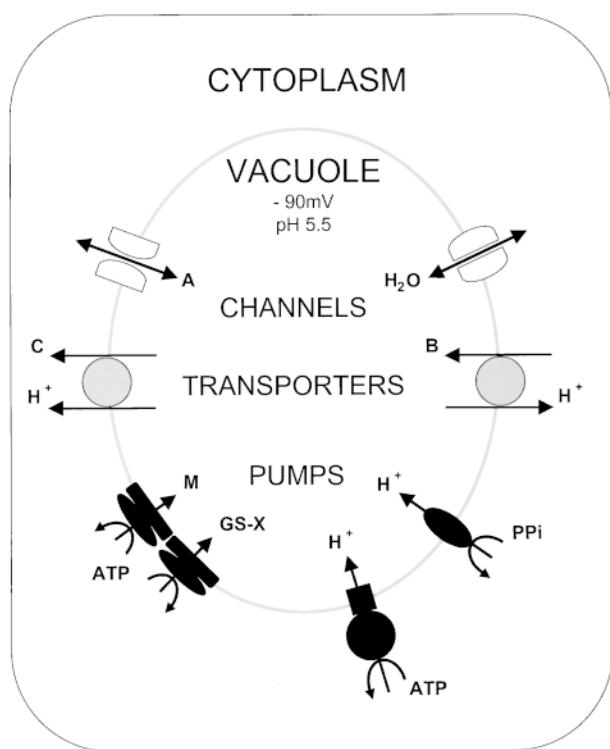


Figure 4. Model of ABC Transporters, H^+ Primary Pumps, H^+ -Coupled Transporters, and Channels in a Simplified Tonoplast.

Glutathione S-conjugate (GS-X) and metabolite (M) transport is achieved by an ABC transporter(s). An electrogenic H^+ -ATPase (V-type) and an H^+ -PPase acidify the vacuole. The proton motive force provides energy for uptake and release of solutes (i.e., cations, anions, and organic solutes, denoted A, B, or C indiscriminately here) across the tonoplast through transporters and channels. Water channels (aquaporins) facilitate the passive exchange of water.

transduction, protoplasmic homeostasis, and regulation of metabolic pathways (Sze et al., 1992).

Recent studies have demonstrated the existence of a group of organic solute transporters, belonging to the ABC superfamily, that are directly energized by MgATP (Rea et al., 1998). These pumps are competent in the transport of a broad range of substances, including sugars, peptides, alkaloids, and inorganic anions. Belonging to the ABC family, the multidrug resistance-associated proteins (MRPs) identified in plants are considered to participate in the transport of exogenous and endogenous amphipathic anions and glutathionated compounds from the cytoplasm to the vacuole. They function in herbicide detoxification, cell pigmentation, storage of antimicrobial compounds, and alleviation of oxidative damage. A role for plant MRPs is also suspected in channel regulation and transport of heavy metal chelates.

CONCLUSIONS AND PERSPECTIVES

Evolutionary perspectives place vacuoles at a central position in the physiological strategies of plants in their environment. In the vast majority of cells from the plant body, vacuoles provide the true *milieu intérieur*. They are responsible for the high cell surface-to-protoplasmic volume ratio required for extensive exchanges of material and information between cells and their environment. In cooperation with the cell wall, they create turgor, which is basic to cell hydraulic stiffness and plant growth. In specialized cells, pigment- and allelochemical-accumulating vacuoles serve as mediators of plant-plant, plant-microorganism, and plant-herbivore interactions. In seeds, vacuoles store proteins to be used for anabolism during seedling growth. The diversity of vacuolar functions parallels a diversity in morphology, biochemistry, and biogenesis.

A number of different intracellular trafficking pathways have already been mapped and provide a structural framework for present concepts in vacuole physiology. The routes are many and varied, but there are significant overlaps, which suggests that although the vacuolar processes serve specific goals, they are all intimately related. Moreover, any one of the compartments from a given trafficking pathway may function so as to be, kinetically and physiologically speaking, "vacuole-like."

Much additional work is needed to characterize vacuoles and their progenitors more precisely by molecular criteria and to adjust recent molecular findings to a structural framework. Plant genetic screens will be useful to identify and characterize genes encoding plant-specific vacuolar functions.

Autophagy, both in the degradative and biosynthetic pathways, arises as a key process in the biogenesis and remodeling of the vacuolar apparatus. It drives the formation of vegetative vacuoles when meristematic cells differentiate, it operates when the vacuolar apparatus switches alternately from vegetative to storage functions, and it is induced by starvation. Many questions are elicited regarding protein trafficking by autophagy. For instance, do autophagic membranes all have the same origin? What triggers the formation, movement, and fusion of the autophagic components? Much has also to be learned about the role of the cytoskeleton in organizing intercompartmental movement of vesicles and shaping vacuoles and their precursors. What are the regulatory mechanisms involved when cells inherit vacuoles from mother cells at mitosis?

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