CHAPTER ONE

Function and Evolution of the Vacuolar Compartment in Green Algae and Land Plants (Viridiplantae)

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Contents

Abstract

Plant vacuoles perform several different functions and are essential for the plant cell. The large central vacuoles of mature plant cells provide structural support, and they serve other functions, such as protein degradation and turnover, waste disposal, storage of metabolites, and cell growth. A unique feature of the plant vacuolar system is the presence of different types of vacuoles within the same cell. The current knowledge about the vacuolar compartments in plants and green algae is summarized and a hypothesis is presented to explain the origin of multiple types of vacuoles in plants.

Key Words: Plant vacuole, Green algae, Protein targeting, Turgor pressure, Autophagy.

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1. Introduction

Plant vacuoles are large single-membrane–bounded compartments within the cytoplasm of a cell that function in several different ways (Marty, 1999). The vacuole is essential for the viability of the plant cell (Rojo *et al.*, 2001). In mature plant cells, vacuoles tend to be very large (80% or more of the cell volume), occupy a central position, and are extremely important in providing structural support, as well as serving functions such as storage, waste disposal, protection, and growth (Marty, 1999). Vacuoles in animal cells, however, tend to be much smaller, and are more commonly used to store materials temporarily or to transport substances.

The central vacuole in a plant cell is enclosed by a membrane termed the tonoplast, which is part of the endomembrane system of the cell (i.e., the vacuole is linked to other compartments of the endomembrane system by vesicular transport) (Surpin and Raikhel, 2004). The large central vacuole develops as the cell matures by fusion of smaller vacuoles, which are derived from the endoplasmic reticulum and/or Golgi apparatus. Because the central vacuole is highly selective in transporting materials through its membrane, the chemical composition of the vacuolar solution (termed the cell sap) differs markedly from that of the surrounding cytoplasm and varies among different cell types (Marty, 1999). For example, some vacuoles contain pigments that give certain flowers their characteristic colors. The central vacuole also contains plant wastes that taste bitter to insects and animals, while developing seed cells use the vacuole as a repository for protein storage. The central vacuole stores salts, minerals, and nutrients; helps in plant growth; and plays an important structural role for the plant. Under optimal conditions, vacuoles are filled with water to the point that they exert a significant pressure against the cell wall (turgor pressure). This turgor pressure is cell specific in regulation (Findlay, 2001) (e.g., in the guard cells of leaf stomata, changes in turgor are used to open and close the stomata). In addition, the turgor pressure helps to maintain the structural integrity of the plant, along with the support from the cell wall and enables the plant cell to grow much larger without having to synthesize new cytoplasm. In most cases, the plant cytoplasm is confined to a thin layer positioned between the plasma membrane and the tonoplast, yielding a large ratio of membrane surface to cytoplasm (Weibe, 1978).

Plant vacuoles are also important for their role in molecular degradation and storage. Sometimes these functions are carried out by different vacuoles in the same cell, one serving as a compartment for breaking down materials (similar to the lysosomes found in animal cells), and another storing nutrients, waste products, or other substances (Marty, 1999).

Here, I will review advances in our understanding of the plant vacuole, concentrating on work published in 2005 and 2006. I will then briefly

discuss the structure and function of vacuoles in green algae. The last section will focus on the origin of the vacuolar system in plants and algae.

2. Structure and Function of Vacuoles in Embryophytes

2.1. Types and functions of vacuoles

It is a common belief that most plant cells contain only the single large central vacuole described above. Therefore, it was a big surprise when Paris *et al.* (1996) showed that two different types of vacuoles were present within the same cell performing different functions (Fig. 1.1A). Since 1996 several studies have shown that plant cells contain multiple types of vacuoles with distinct functions (Di Sansebastiano *et al.*, 2001; Epimashko *et al.*, 2004; Jauh *et al.*, 1998, 1999; Park *et al.*, 2004). Up to three separate distinct types of vacuoles have been reported within a single plant cell (Jauh *et al.*, 1999). Generally, vacuoles are divided into two categories. Lytic vacuoles (see LVs, Fig. 1.1A) are acidic compartments and are rich in hydrolases. LVs are considered as equivalent to the animal lysosome and are recognized by the presence of γ-TIP (the γ-isoform of tonoplast intrinsic proteins, a member of the large glyceroaquaporin protein family present in plants). Protein storage vacuoles (see PSVs, Fig. 1.1A) are most often found in storage organs and are characterized by the presence of α-TIP (Jauh *et al.*, 1999), but have recently also been reported in mesophyll cells (Park *et al.*, 2004). The presence of separate vacuoles of distinct function in seed plants is in marked contrast to animal and fungal cells that contain only lysosomes or a single vacuole (see also the discussion of this question by Robinson *et al.*, 2005).

In the past 2 years, progress has been made in understanding most aspects of vacuolar function. The following is a brief summary of major recent findings regarding vacuolar function.

2.1.1. Turgor

The structural importance of the plant vacuole is related to its ability to control turgor pressure. Turgor pressure dictates the rigidity of the cell and is associated with the difference between the osmotic pressure inside and outside of the cell. The role of aquaporins in water relations of the vacuole is a hot topic in plant research. Since the progress in this field has been reviewed several times in the past years, the reader is referred to the reviews by Hachez *et al.* (2006) and Luu and Maurel (2005) for further information.

2.1.2. Storage

Plant vacuoles store a large variety of chemical compounds. The protein targeting of storage protein to specialized vacuoles (protein storage vacuoles) and the processing inside the vacuoles are discussed in Section 2.2.

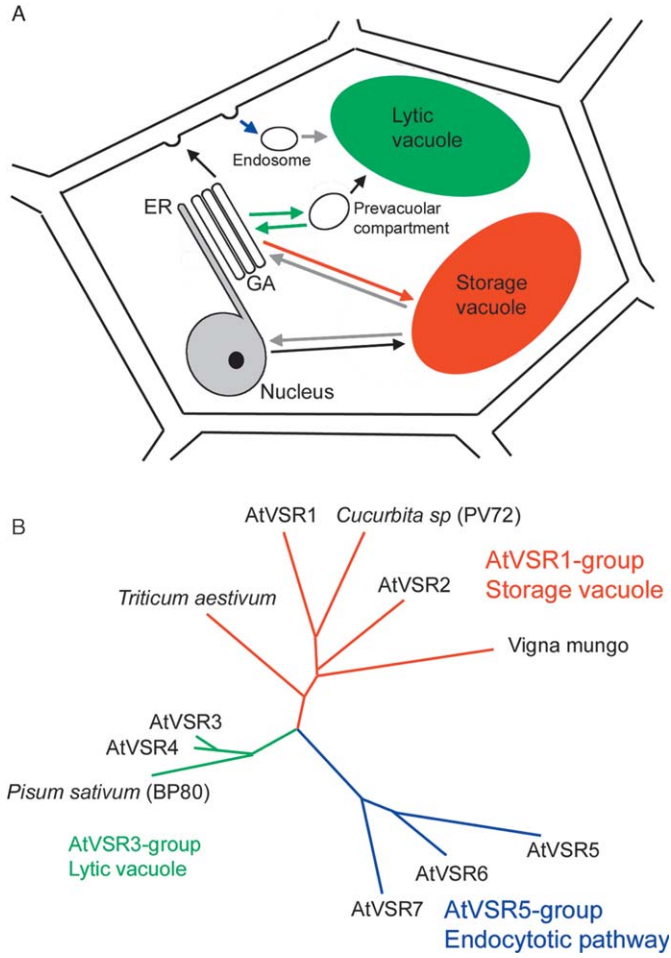

Figure 1.1 (A) The endomembrane system of a plant cell. The vesicular transport pathways to the vacuole are shown. Uncharacterized pathways expected to be present are in gray. Pathways possibly involving vacuolar-sorting receptors (VSRs) are colored according to the suggested functions (Masclaux *et al.*, 2005) for the three phylogenetic groups of *Arabidopsis* VSRs: green, lytic vacuole; red, protein storage vacuole; and blue, endocytotic pathway. (B) Phylogenetic relationships of VSRs from *Arabidopsis* and a few other selected angiosperms. The color coding is the same as in (A). (Modified from Masclaux *et al.* [2005].)

In addition, the large central vacuole is known to store several other small molecules. Stored metabolites and chemicals can either serve as a cellular pool, on which the cell can rely during starvation, or as a detoxification step to prevent interference of chemicals with cellular function.

It has long been known that nitrate, the principal nitrogen source of most plants, can accumulate in large quantities in certain plants (e.g., of the

families Chenopodiaceae, Poaceae, Brassicaceae, and Asteraceae) (Martinoia *et al.*, 1981). Most of the nitrate is stored in the vacuole. However, the mechanism of uptake into the vacuole has remained elusive for years. More recently, De Angeli *et al.* (2006) provided evidence that nitrate is transported into the vacuole using a proton antiport mechanism and localized a member of the voltage-gated chloride channel protein family (AtCLCa) to the tonoplast, confirming its involvement in nitrate transport as earlier suggested (Geelen *et al.*, 2000).

Several other vacuolar carrier systems have already been characterized at the molecular level, and recent work has added to this list, including Zn (Elbaz *et al.*, 2006), Al (Kochian *et al.*, 2005), Na (Yamaguchi *et al.*, 2005), Mn (Pittman, 2005), Ca (Pittman *et al.*, 2005), Fe uptake systems (Kim *et al.*, 2006), and a novel monosaccharide transporter (Wormit *et al.*, 2006).

Detoxification of xenobiotics generally occurs in four steps: activation of a xenobiotic, formation of a GSH-S-conjugate (GSX), sequestration (in plants: into the vacuole), and degradation of GSX (Sandermann, 1994). In mammals GSX is degraded by γ-glutamyltransferases (GGTs) and L-cysteinglycinyldipeptidase (DPase) reactions (Meister and Anderson, 1983). Whereas the first three steps are well characterized in plants (Foyer *et al.*, 2001; Rea, 1999), degradation of GSX by GGT and DPase has not been demonstrated in plants so far. Nakano *et al.* (2006) have now demonstrated the presence of GGT and DPase activity in isolated vacuoles from radish cotyledons suggesting that the complete pathway for detoxification is conserved between animals and plants.

The proteome of the tonoplast from *Arabidopsis thaliana* has been characterized (Carter *et al.*, 2004; Sazuka *et al.*, 2004; Shimaoka *et al.*, 2004; Szponarski *et al.*, 2004). In addition to proteins known to be present (V-ATPase, H^+ transporting PPase, TIPs), several proteins with unknown or unexpected functions were detected. Additional work will be required to analyze the relevance of their presence in the isolated tonoplast fractions. However, the list of tonoplast proteins is far from complete, as revealed by a new proteomic study. Endler *et al.* (2006) identified 40 additional proteins in the tonoplast fraction isolated from barley leaves that were not detected in the four proteomic studies of the tonoplast of *A. thaliana*. Among the new proteins identified by Endler *et al.* (2006) is the first tonoplast sucrose transporter (HvSUT2). *Arabidopsis* mesophyll cells possess a homologue of HvSUT2 (AtSUT4) on their tonoplast membrane. Sucrose is mainly stored in the vacuole of plant cells. In leaves, sucrose is transported to the vacuole during the light period. At night, sucrose is then transported to the phloem for transport to other tissues (Kaiser and Heber, 1984).

Salt stress is a major problem in agriculture. About 20% of the world's cultivated lands are affected by salinity (Chrispeels and Sadava, 2003). To cope with high salinity, halophytes have evolved mechanisms to protect their cells from the detrimental effects of salts on plants. Among other

adaptations (increased cytosolic concentrations of compatible solutes), halophytes sequester sodium to vacuoles to increase vacuolar osmolarity and keep sodium away from the sites of metabolism. Sodium is transported into vacuoles using an Na^+/H^+ antiporter. The Na^+/H^+ antiport is driven by the electrochemical proton gradient generated by V-ATPase and V-PPase. From these two the latter enzyme activity has been shown to be increased during salt stress in *Salicornia bigelovii* (Parks *et al.*, 2002). More recently, Guo *et al.* (2006) cloned the V-PPase from the halophyte *Suaeda salsa*. When the V-PPase from *S. salsa* was expressed in *Arabidopsis,* increased salt and drought tolerance were observed (Guo *et al.*, 2006).

2.1.3. Autophagy and vacuole-mediated cell death

Autophagy is the nonselective uptake of large portions of the cytosol and/or organelles by encapsulating cellular material with membranes, transporting the material to the vacuole, and degradation in the vacuole. Autophagy is an important mechanism for protein turnover, as well as a universal reaction to cell starvation. The process is best characterized in yeast (see Klionsky *et al.*, 2003 for a recent list of autophagy-related genes). Autophagic transport to the vacuole occurs by two morphological distinct but mechanistically overlapping pathways: microautophagy and macroautophagy (Reggiori and Klionsky, 2002). In microautophagy the material to be degraded is directly taken up by the lysosome/vacuole. In macroautophagy the material to be degraded is first engulfed by membranes separate from lysosomes/vacuoles and then transported to the vacuole. Two different autophagic pathways have been demonstrated to occur in plant cells (Toyooka *et al.*, 2001). Degradation of starch granules in mung bean cotyledons is clearly similar to microautophagy (Toyooka *et al.*, 2001), whereas the uptake of cytosol and mitochondria involved a mechanistically different second pathway. The presence of a macroautophagic pathway in plants has recently been demonstrated by Toyooka *et al.* (2006). In addition to a role in mobilization of nutrients during germination, autophagy constitutively takes place in root tips (Inoue *et al.*, 2006) and is also involved in other processes. Thompson *et al.* (2005) reported that autophagy plays an essential role in nutrient recycling in *Arabidopsis*. Plants lacking the autophagic pathway display early senescence and are hypersensitive to carbon and nitrogen starvation (Thompson *et al.*, 2005). Stroma proteins of the chloroplast have been shown to be transported to the vacuole (Chiba *et al.*, 2003); however, whether this involves autophagy or another mechanism to be discovered is an open question .

Plants exhibit programmed cell death (PCD) during plant development (e.g., floral organs, Rogers, 2006) or as a response to pathogens. Although some mechanisms underlying PCD are thought to be conserved between plants and animals, a key feature of PCD in animals is the dependence on caspase protease activity. Up to now, the presence of caspase activity in plants has remained elusive. Hatsugai *et al.* (2004) reported that a vacuolar processing

protease (VPE), which is structurally unrelated to caspase, has a caspase-1 activity (see Hatsugai *et al.*, 2006 for a detailed comparison of VPE with caspases). Furthermore, this enzyme is essential for virus-induced responses that involve PCD (Hatsugai *et al.*, 2004), indicating that PCD in plants is mediated by the vacuole. Recently it has been hypothesized that an autophagic pathway is required for executing PCD (Seay and Dinesh-Kumar, 2005).

2.2. Structure and development of vacuoles

Fluorescent probes (e.g., green fluorescent protein [GFP]) have been used for more than a decade to address biological questions. However, the focus of most studies using GFP-tagged vacuolar proteins (e.g., Czempinski *et al.*, 2002; Kataoka *et al.*, 2004; Kutsuna and Hasezawa, 2002; Mitsuhashi *et al.*, 2000; Reisen *et al.*, 2003; Ueoka-Nakanishi *et al.*, 2000; Yano *et al.*, 2004) was to localize the protein and not to study the dynamics or development of the vacuole. Only a few studies have addressed the structure and dynamics of the vacuolar system.

The structure of the vacuolar system of germinating pollen was investigated by Hicks *et al.* (2004) using a γ-TIP-GFP construct. In germinating pollen the vacuolar system consists of elongated (tubular) vacuoles with highly mobile cytoplasmic invaginations. Hicks *et al.* (2004) also investigated the effect of the vacuoless1 mutation *(vcl1)* on the structure of the vacuole in the male gametophyte. *Vcl1* was shown to be essential for biogenesis of the vacuole in the embryo. Inactivation of both copies of VCL1 caused the accumulation of small vesicles and autolysosomes in the cells and led to lethality at the torpedo stage of embryogenesis (Rojo *et al.*, 2001). Surprisingly, *vcl1* did not affect the vacuolar structure in the male gametophyte, although it affected the fertility of the male gametophyte (Hicks *et al.*, 2004).

The structure of the vacuolar system is also affected by environmental signals. Irani and Grotewold (2005) demonstrated that light affected the morphology of the vacuolar system in BMS (Black Mexican Sweet, a maize cell line) cells. In maize anthocyans are synthesized after induction by light. Using a genetically engineered cell line expressing the biosynthetic enzymes constitutively (using the CaMV 35S promoter) and leading to accumulation of anthocyans in the dark, changes in cell pigmentation in BMS cells upon transfer to light were observed (Irani and Grotewold, 2005). Subsequently, it could be demonstrated that the changes in cell pigmentation were not due to different cellular concentrations of anthocyans. Instead, several small anthocyan-accumulating vacuoles fused to a large central vacuole upon transferring BMS cells to light (Irani and Grotewold, 2005), indicating that light is regulating the structure of the vacuolar system in this cell line.

The response of the vacuole to osmotic changes is well known (plasmolysis and deplasmolysis). While the tonoplast protein complexes have been well

studied during this process, the tonoplast itself is less well described. Reisen *et al.* (2005) have determined the three-dimensional structure of the vacuolar system upon (acclimation to) osmotic stress. During plasmolysis the tonoplast of the central vacuole folds, but no vacuolation or vesiculation of the vacuole was observed. When the cells were allowed to acclimate to the osmotic stress, the large central vacuole was converted into a complex vacuolar network with an increased tonoplast surface area upon osmotic stress (Reisen *et al.*, 2005). Mimicking desiccation, polyethylene glycol (PEG) treatment of cells led to spherical structures composed of tonoplast material inside the vacuoles. Again no vacuolation or vesiculation of the tonoplast was observed as reported earlier by Chang *et al.* (1996) and others.

Actin filaments have been shown to be important for maintaining the structure of the large vacuoles in tobacco BY-2 cells (Higaki *et al.*, 2006). Disruption of the actin cytoskeleton caused the formation of small spherical vacuoles (Higaki *et al.*, 2006). Changes in the structure of the vacuolar system are also important for the stomata opening in plants. Gao *et al.* (2005) reported that in the stomata cells of *Vicia faba* the vacuolar system consists of several small vacuoles when the stomata are closed. During opening the small vacuoles fuse to large vacuoles. Furthermore, a mutation in the SGR3 gene, which is involved in vacuolar fusion, leads to retardation of the stomata opening, indicating that vacuolar fusion is important for the stomata opening process.

As an alternative approach to studying the structure and function of the vacuole in living cells, Dubrovsky *et al.* (2006) suggested using Neutral Red as a probe to investigate the structure of vacuoles with laser-scanning microscopy.

2.3. Protein targeting to vacuoles

Protein trafficking to vacuoles in land plants is highly complex, due to multiple types of vacuoles (lytic and storage vacuoles) occurring in plants. A comparison with the animal and yeast lysosomal/vacuolar system was recently presented by Robinson *et al.* (2005). Recent research has concentrated on the vacuolar-sorting receptors (VSRs). Soluble vacuolar proteins bind to the VSR in the Golgi complex and are transported to the vacuole. Upon arrival at the vacuole or a prevacuolar compartment, the complex of cargo protein and VSR dissolves and the VSR are retrieved to the Golgi complex. So far vacuolar-sorting receptors have been investigated in detail only from pumpkin (PV72), pea (BP-80), and *Arabidopsis* (AtELP). All are type I integral-membrane proteins with EGF-like motifs in their lumenal domain. Vacuolar sorting receptors such as BP-80 are concentrated on post-Golgi membranes (Li *et al.*, 2002) and are constitutively retrieved from the prevacuolar compartment to the Golgi apparatus by a saturable mechanism (Da Silva *et al.*, 2005). The targeting of BP-80 was investigated by a mutational analysis (Da Silva *et al.*, 2006). Da Silva *et al.* (2006) showed

that the several amino acid motifs in the cytoplasmic tail and the transmembrane domain are required for proper targeting of BP-80. Pea BP-80 and *Arabidopis* AtELP are thought to be involved in transport to the lytic vacuole. In *Arabidopsis,* VSRs form a small protein family with seven members. Masclaux *et al.* (2005) suggested that the three phylogenetic branches observed for the seven VSRs from *Arabidopsis* (Masclaux *et al.*, 2005; Paris and Neuhaus, 2002; Shimada *et al.*, 2003) reflect different functions of the VSRs; they have tentatively assigned the three branches to the lytic, storage vacuole, and a putative endocytotic pathway (Fig. 1.1). However, Park *et al.* (2005) have identified AtRMR1 (a unique receptor-like protein of the ReMembR-H2 [RMR] protein family) as a VSR involved in transport to the storage vacuoles. This suggests that either structurally different receptors are involved in the same transport pathway or the interpretation of VSR phylogeny by Masclaux *et al.* (2005) is not correct.

In animals and fungi, lysosomal/vacuolar trafficking depends on the retrograde transport of the vacuolar-sorting receptor to the Golgi complex. Several proteins forming a retromer complex have been implicated in this process. Now Shimada *et al.* (2006) report the characterization of the first plant mutant in a retromer component. As might be expected the mutant fails to accumulate 12S globulin and 2S albumin in the storage vacuole. Instead these proteins are secreted. Apparently, the lack of a vacuolar-sorting receptor in the Golgi complex, due to failure to recycle the receptor, causes the misrouting of these proteins (Shimada *et al.*, 2006).

Other components of the complex machinery delivering proteins to the vacuoles were recently characterized. Proteins containing an epsin N-terminal homology (ENTH) domain have been identified as playing a critical role in various vesicular transport steps. The ENTH domain specifically binds phosphatidylinositols (different ENTH domains have a different lipid specificity) and is thought to be responsible for targeting these proteins to specific compartments and to assist in the formation of clathrin-coated vesicles by introducing curvature to the membrane (Legendre-Guillemin *et al.*, 2004). Now Song *et al.* (2006) report that *Arabidopsis* epsin 1 interacts with clathrin and the adaptor-1 complex and plays an important role in vacuolar trafficking of soluble proteins. Other proteins involved in vacuolar trafficking currently also being investigated include a GTPase-activating protein in rice (Heo *et al.*, 2005) and the role of specific syntaxin isoforms in the vacuolar system (Foresti *et al.*, 2006).

Although the basic characteristics of plant vascular sorting signals (VSS) were worked out several years ago (Vitale and Raikhel, 1999), identifying N-terminal, internal, and C-terminal VSS in several proteins, research on vacuolar-sorting signals in vacuolar proteins is still going on. VSS were recently investigated in soybean 11S globulin (Maruyama *et al.*, 2006). Similar to the situation for BP-80 (see previous), multiple sorting also exists in the 11S globulin of soybean. In contrast, targeting of proConA to the

vacuole depended on a new nine-amino acid-containing C-terminal propeptide, when proConA was expressed in tobacco (Claude *et al.*, 2005). In addition, a recent investigation of VSS function in proricin showed that the position of the VSS is important for correct function of the VSS to the storage vacuole (Jolliffe *et al.*, 2003). Most proteins are transported via the Golgi apparatus to the vacuoles; however, some storage proteins follow other routes—the direct endoplasmic reticulum (ER) to the vacuole pathway or the autophagy pathway. Oufattole *et al.* (2005) have now identified an amino acid sequence PIEPPPHH directing a membrane protein to the ER to the vacuole pathway. They showed that transport depends on a putative receptor AtSRC2, which binds the sequence PIEPPPHH and is required for internalization of the ER-derived transport vesicle into the vacuole (Oufattole *et al.*, 2005).

Many plant proteins undergo proteolytic processing during their transport from the Golgi to the vacuoles. Otegui *et al.* (2006) have now analyzed this process in detail in *Arabidopsis*. They show that storage proteins and processing enzymes are packaged in separate vesicles in the Golgi. Both vesicles seem to fuse into a prevacuolar compartment, where the processing of the 2S albumin starts.

3. Structure and Function of Vacuoles in Green Algae

3.1. Types and functions of vacuoles

It was 16 years ago that Domozych (1991) reviewed this topic in this series. At that time he wrote: "Little is known about the 'non-contractile' or cytoplasmic vacuoles of green algae, especially about their origins and functions. Because of their common occurrence in most green algae, it may be assumed that they are important in turgor control or waste storage, similar to the vacuole in higher plants"(Domozych, 1991). In agreement with this statement the transport capacities of the tonoplast membranes in green algae were found generally to be similar to land plants (Bethmann *et al.*, 1995; Heidecker *et al.*, 1999, 2003a,b; Mimietz *et al.*, 2003; Raven, 1989). Progress in this area is still slow; however, in the following I want to highlight some recent progress in this research area, but it is not the aim of this review to summarize all the work published since Domozych published his review in 1991.

3.1.1. Contractile vacuole

The structure of contractile vacuoles has now been described in some detail in the two chlorophyte algae *Chlamydomonas* (Luykx *et al.*, 1997a,b) and *Scherffelia* (Becker and Hickisch 2005, Fig. 1.2). The structure of the

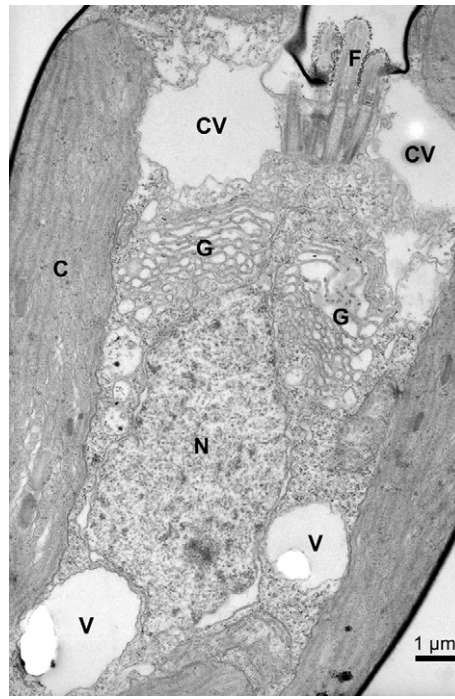

Figure 1.2 Electron micrograph of a *Scherffelia dubia* cell. The contractile vacuole (CV) and the polyphosphate storing vacuoles (V) are indicated. C, chloroplast; F, flagellum; G, Golgi stack; N, nucleus.

contractile vacuole in both organisms is very different. In *Chlamydomonas,* the large round vacuole typically observed shortly before it discharges its content into the medium develops from small vacuoles by membrane fusion (Luykx *et al.*, 1997b). In contrast, in *Scherffelia,* the large round vacuoles develop from a membranous reticulum (Becker and Hickisch, 2005). From this and other work (Allen and Naitoh, 2002; Patterson, 1980) it is now clear that there is considerable variation in the structure of the contractile vacuole in protists. Whether the CVs use the same or similar mechanisms for their function cannot be answered today. Further work is required on structurally different CVs to address this problem.

3.1.2. Other vacuoles

Many algae contain intracellular granules, which stain with basic dyes. These granules have been referred to as volutin or metachromic granules, and it is generally agreed that the volutin granules are storage vacuoles containing polyphosphate. Recently, this has been confirmed by EDAX analysis and

biochemical analysis of isolated granules from *Chlamydomonas* (Komine *et al.*, 2000). Since these polyphosphate-containing vacuoles possess a proton pumping pyrophosphatase (Ruiz *et al.*, 2001), V-ATPase (Ruiz *et al.*, 2001) and acid phosphatase (Matagne *et al.*, 1976), the polyphosphate-containing vacuoles of *Chlamydomonas* represent the lytic compartment in *Chlamydomonas*. These vacuoles are apparently also involved in degradation of plastidic proteins (Park *et al.*, 1999).

3.2. Development of vacuoles

In many large unicellular or multicellular algae, the cells contain a large central vacuole. Whereas the transport capacities of the tonoplast membrane have often been investigated, little is known about the development and dynamics of the large vacuoles in algae. Changes in the structure of the vacuolar system during the life cycle have been investigated only in *Acetabularia* (Ngo *et al.*, 2005) using Neutral Red and light microscopy. During development the large central vacuole increases in size from 10 μm to 35 mm. Local application of the dye was used to investigate the connectivity of the large central vacuole within the thallus. Interestingly, the dye moved at different rates through different regions of the central vacuole indicating that the internal structure of the various regions of the central vacuole are different. It was concluded that the central vacuole of *Acetabularia* is a ramified polar organelle with a gellike sap. The morphology of the central vacuole is actively remodeled during development (Ngo *et al.*, 2005).

Cell growth is among other factors controlled by the TOR kinase in eukaryotes (Inoki *et al.*, 2005). In contrast to land plants and similar to other eukaryotes, the TOR kinase of *Chlamydomonas* is inhibited by rapamycin (Crespo *et al.*, 2005). Treatment of *Chlamydomonas* cells with rapamycin affected the structure of the vacuolar system possibly due to enhanced autophagy (Crespo *et al.*, 2005), suggesting that the structure of the vacuolar system is also regulated during cell growth in small unicells.

Environmental parameters (e.g., carbon dioxide, heavy metals) affect the number and function of vacuoles. Sasaki *et al.* (1999) reported an increase in the number of vacuoles and the activity of tonoplast proteins in *Chlorococcum littoreale* when cells were incubated under extremely high carbon dioxide concentrations. In another study (Nishikawa *et al.*, 2003) it was found that at high concentrations of heavy metals (especially cadmium) the number and volume of vacuoles increased in *Chlamydomonas acidophilum*.

Nothing is known about protein trafficking to the vacuole in green algae.

4. Evolution of Vacuolar Compartments in Plants

Before discussing the evolution of the vacuolar system in plants, let me briefly summarize our current knowledge on the evolution of plants (Fig. 1.3). At the present time, there is a broad consensus that primary plastids evolved only once from a cyanobacterium that was taken up by a eukaryotic flagellate. Glaucophytes (Glaucocystophyceae), red algae (Rhodoplantae), and green algae and land plants (Viridiplantae), which dominate many of today's ecosystems, are the descendants of this singular event (Keeling, 2004). The Viridiplantae are grouped into two phyla: the Chlorophyta, which include the Chlorophyceae, the Ulvophyceae, the Trebouxiophyceae, and most prasinophytes (scaly green flagellates); and the Streptophyta, which include a small group of freshwater algae known as Charophyceae, the scaly flagellate *Mesostigma,* and the embryophytes (Lewis and McCourt, 2004). Most likely, the Chlorophyta and Rhodophyta evolved in a marine environment, whereas the Streptophyta originated in a freshwater or brackish habitat (Falkowski *et al.*, 2004; Simon *et al.*, 2006).

The vacuole is part of the eukaryotic endomembrane system, and many of the molecular components required for the biogenesis and maintenance of the endomembrane system are very well conserved between different eukaryotes (Becker and Melkonian, 1996). Therefore, the last common ancestor of the Viridiplantae inherited a typical eukaryotic endomembrane system. A general evolutionary trend in plants is the formation of a large central vacuole that allows the cell volume to increase without the need to invest in cytoplasm and other organelles. However, this is not a development specific to plants, as a large central vacuole is also found in various other organisms, (e.g., fungi and heterokont algae). The formation of a large central vacuole is observed in several phylogenetic groups within the Viridiplantae (e.g., chlorophytes, ulvophytes, and charophytes), which evolved independently from a unicellular flagellate, and, therefore, the central vacuole probably evolved several times independently even within the Viridiplantae.

In many plants and green algae the vacuole adapted to specialized functions (e.g., storage of special compounds such as anthocyanins in the leaf epidermis of many angiosperms). In other cases vacuoles serve the same functions in plants and green algae; however, they use different mechanisms to perform this function. One example of this phenomenon is the storage of phosphorus in the vacuole. In algae, phosphorus is generally stored as polyphosphate, whereas in angiosperms, phosphorus is stored as phytic acid in the vacuole (Mitsuhashi *et al.*, 2005).

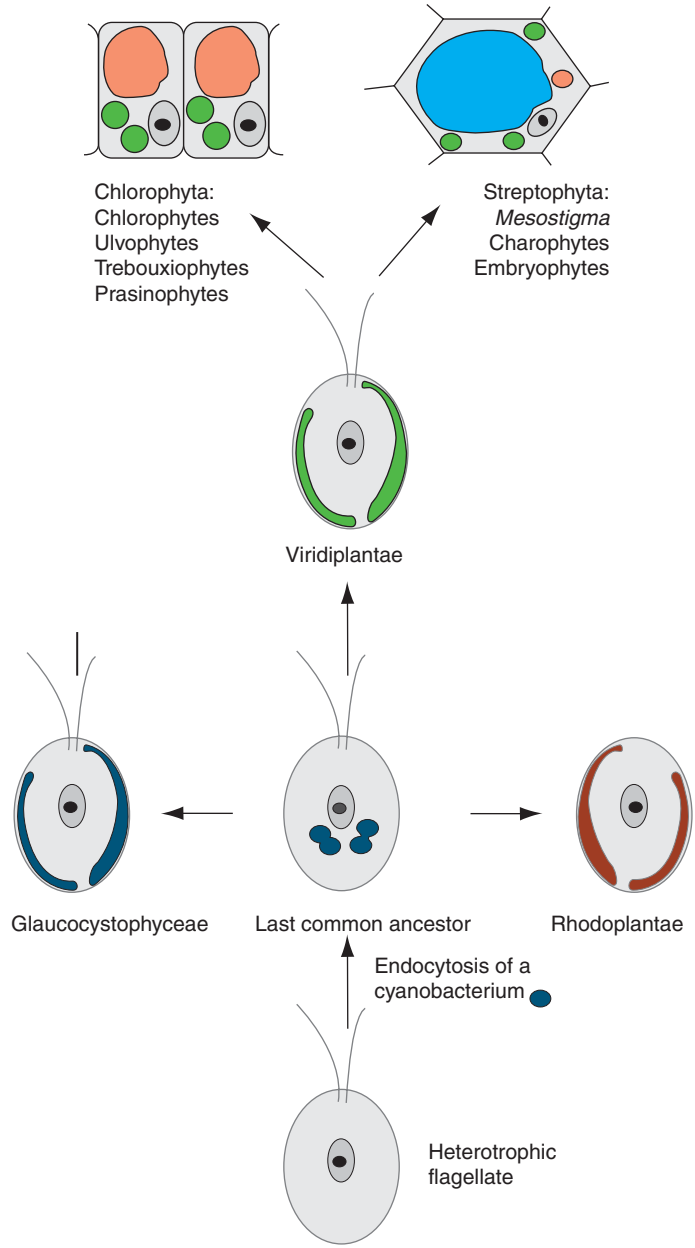

Figure 1.3 Evolution of plants. Primary plastids evolved once from a cyanobacterium taken up by a heterotroph flagellate. Glaucophytes, red algae, and green algae (and embryophytes) are the descendants of this unique event. Within the green algae two major evolutionary lines are observed: the Chlorophyta, which include most green algae (e.g., *Chlorella*, *Chlamydomonas*, *Ulva*); and the Streptophyta, which include *Mesostigma*, a small group of freshwater algae known as the Charophyceae (e.g., Charales, Coleochaetales, and desmids), and the embryophytes.

As previously indicated, the major differences between the vacuolar system of plants and the vacuoles or lysosomes of fungi and animals is the presence of different types of vacuoles within a single plant cell. How can we explain this difference? It is important to note that seed plant cells are not unique in containing separate vacuoles with distinct functions. Many freshwater cell wall-less protists contain a contractile vacuole (CV) involved in osmoregulation and an acidic (lytic?) vacuole that might also serve a storage function (e.g., polyphosphate in algae) (see Fig. 1.2). Life on earth evolved in a marine environment. Organisms from several evolutionary lines invaded the freshwater habitat and were faced with the problem of water uptake by osmosis in a freshwater environment. Faced with a hypotonic medium, CVs evolved several times, most likely independently, using the same basic mechanisms but structurally unique solutions to the problem (Allen and Naitoh, 2002; Patterson, 1980). Some osmotolerant freshwater protists lose their contractile vacuoles when transferred into a hypertonic medium (Allen and Naitoh, 2002). Thus, protists seem to be able to "switch between a CV-containing and CV-less life style" (Fig. 1.4). The evolution of multicellularity took place several times on earth (e.g., animals, fungi, streptophytes [charophyte algae and land plants], red algae, brown algae). In this context it is remarkable that within the above-mentioned groups only

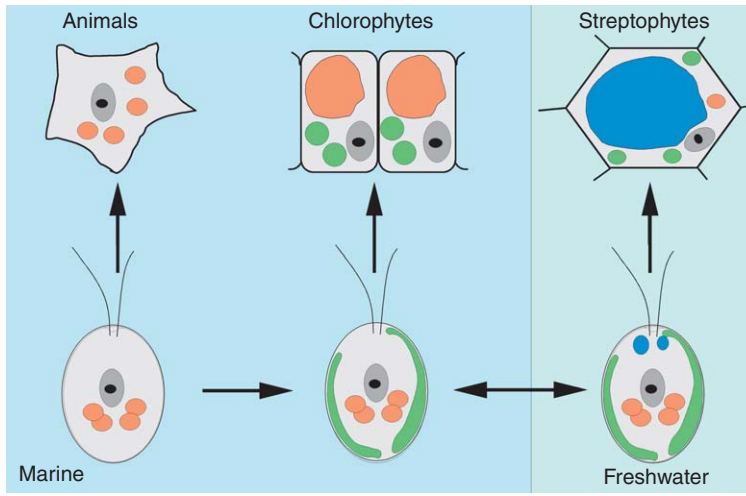

Figure 1.4 Evolution of the vacuole. Life started in a marine environment. When protists invaded the freshwater habitat contractile vacuoles developed. Streptophytes are the only major evolutionary line that evolved in a freshwater environment. Freshwater protists and seed plants are the only known organisms having different types of vacuoles. Green, chloroplasts; orange, acidic (lytic) vacuole (lysosome); blue, vacuole involved in osmoregulation; gray, nucleus with nucleolus. For simplicity, heterotroph freshwater protists have been omitted.

streptophytes made the transition to multicellularity in a freshwater environment (however, note that the question of whether fungi evolved in a marine or freshwater habitat is still not settled [James *et al.*, 2006]). Therefore, I propose that the separate vacuoles found in seed plants may be derived from the different types of vacuoles present in the last cell wall-less ancestor of the streptophyte algae (see Fig. 1.4). How can we obtain proof for such a hypothesis? Distinct types of vacuoles in seed plants can be identified by the TIP isoform present on the tonoplast membrane: α-TIP is associated with the protein storage type of vacuoles (Park *et al.*, 2004), γ-TIP is associated with the lytic type of vacuoles (Jauh *et al.*, 1999), and δ-TIP is found in pigment-containing vacuoles (Jauh *et al.*, 1998). TIPs belong to the aquaglyceroporin protein superfamily and represent a plant-specific subfamiliy. It has been suggested that diversification of plant aquaporins into the PIP, TIP, NIP, and SIP subfamilies preceded the divergence of bryophytes and tracheophytes (Borstlap, 2002). However, preliminary evidence indicates that differentiation of TIP isoforms might have occurred later as all moss-specific TIPs form an independent lineage in phylogenetic trees and do not cluster together with seed plant TIPs (Borstlap, 2002). In addition, the genome (http://genome.jgi-psf.org/chlre2/chlre2.home.html) of the unicellular freshwater chlorophyte *Chlamydomonas* probably contains only one functional aquaporin, and green algal genomes and ESTs do not contain any evidence of aquaporins of the TIP subfamily (unpublished observations). Thus, TIPs might not be the right marker to address this question.

Every cell maintaining two different types of vacuoles is faced with the problem of protein targeting of vacuolar proteins to their different destinations. In seed plants proteins use different targeting signals and pathways for transport to a lytic or a protein storage type of vacuole (Vitale and Galili, 2001). So far, the latter has been reported only for seed plants. Whether these pathways are present in other streptophyte groups has never been addressed to my knowledge. If the hypothesis presented above is correct, the two pathways might be conserved within streptophytes. To address this question I tried to detect homologues of VSRs (and RMRs), storage proteins, and the plant-specific thiol protease aleurain within the Viridiplantae using BLAST analysis (Table 1.1). Homologues of VSRs and an aleurain type of thiol protease were found in all green algae, whereas the RMR-type receptor appears to be restricted to embryophytes. Because significant hits outside the Viridiplantae were not observed for any of these molecular markers, the molecular markers represent true innovations of the Viridiplantae. Aleurain and its sorting receptor VSR date back before the separation of the chlorophyte and streptophyte evolutionary line, and the RMR-type receptor, which is involved in traffic to the PSV, is clearly present in liverworts and bryophytes and might indicate the presence of PSV in embryophytes. Whether green algae contain a PSV is currently an open

Table 1.1 Evolution of the vacuolar system[a]

Protein	Chlorophytes	Streptophyte algae[b]	Bryophytes[b]	Ferns[b]
VSR	+	+	+	+
RMR	−	−	+	+
Aleurain	+	+	+	+
Storage proteins[c]	−	−	−	+

[a] The results of a BLAST analysis using angiosperm storage proteins, aleurain, VSR, and RMR-type receptor proteins (NCBI and JGI database using an expect threshold $<e^{-10}$). +, protein with significant similarity found; −, no protein with significant similarity detected.
[b] No complete genome is available; only ESTs were analyzed.
[c] Using phaseolin, 11S globulin, 7S globulin, and sporamin from angiosperms as query.

question. A RMR-type receptor is missing so far in the published ESTs from green algae, and we have not detected an RMR-type receptor in our 10,000 ESTs from two other streptophyte algae, *Klebsormidium* and *Coleochaete* (unpublished observations). However, sequences showing significant homology to VSRs from the streptophyte *Coleochaete* and from the chlorophytes *Ostreococcus* (two strains) and *Prototheca wickerhamii* cluster in preliminary phylogenetic analyses with the AtVSR1 group, which has been suggested to be involved in transport to the PSV (Masclaux *et al.*, 2005). The genome of the two *Ostreococcus* strains has been sequenced completely (Derelle *et al.*, 2006, and http://genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html), and only a single protein with strong similarity to AtVSR1 was found in both strains. Therefore, sorting of vacuolar proteins with an AtVSR1-like protein probably evolved early during the evolution of green algae. Interestingly, ESTs from streptophyte algae (*Closterium* and *Klebsormidium*) as well as ESTs from various bryophytes, liverworts, and ferns cluster with the AtVSR5–7 proteins, which have been suggested to be involved in sorting in the endocytotic pathway (Masclaux *et al.*, 2005). However, because the total number of different VSR proteins is not known for any of these organisms, it is not clear whether this result is significant.

5. Concluding Remarks

The plant vacuoles continue to be a fascinating topic. Although much progress has been made in our understanding of many important vacuolar functions, key questions regarding the biosynthesis and evolution of the plant vacuolar system have still not been completely answered. However, it

is clear that the growing number of genomes and ESTs from green algae and lower embryophytes will help to unravel these problems.

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