Dynamic Organization of Vacuolar and Microtubule Structures during Cell Cycle Progression in Synchronized Tobacco BY-2 Cells

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Introduction

Vacuoles of plant cells are the largest of compartments and multifunctional organelles, and serve for general cell homeostasis. They function in storage and osmoregulation and, like lysosomes of animal cells, contain many hydrolytic enzymes for digestive processes. The functions of vacuoles, which are thought to be dynamic rather than static, are regulated both in time and space on the basis of the developmental stage and environmental condition (Wink 1993, Marty 1999).

As vacuolar functions depend on a series of soluble and membrane-bound proteins, the various proteins are transported to the vacuoles. Most of them are synthesized and translocated into the endoplasmic reticulum (ER) and then transported to the vacuoles through the secretory pathway. Over the last decade, the analysis of gene products modified in yeast vacuolar mutants has also provided new insights into intracellular trafficking in plant cells. For example, various proteins known to be involved in trafficking, such as clathrin, the coat-mer subunits of coat protein I vesicles, as well as soluble N-ethylmaleimide-sensitive factor attachment proteins, and many small GTP binding proteins have been identified in plant cells (Sanderson and Raikhel 1999). In contrast to yeast, which has a single lytic vacuole, higher plant cells have several biochemically and structurally distinct types of vacuoles that are classified as either lytic vacuoles or protein storage vacuoles (PSVs) (Herman and Larkins 1999, Jiang and Rogers 2001). The vacuoles can be distinguished by the presence of different tonoplast intrinsic proteins in their limiting membranes (Paris et al. 1996, Jauh et al. 1998). Additionally, these two types of vacuoles can be dynamically interchanged during different cellular developmental processes. For example, during germination and seedling growth, some independent PSVs can fuse and form into a single lytic vacuole. Recent evidence also suggests that some plant cells may contain multiple types of vacuoles (Paris et al. 1996, Di Sansebastiano et al. 1998, Jauh et al. 1998, Jauh et al. 1999). Moreover, vacuolar transport pathways and vacuolar targeting signals are not identical among the vacuolar proteins. Some proteins are transported to the vacuoles by the clathrin-coated vesicles via the prevacuolar compartment or by the Golgi-derived dense vesicles (Hohl et al. 1996). Other proteins are delivered to the vacuole bypassing the Golgi apparatus in precursor-accumulating (PAC) vesicles (Hara-Nishimura et al. 1998) or dark intrinsic protein (DIP) organelles (Jiang et al. 1999).

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Abbreviations: CLSM, confocal laser scanning microscopy; ER, endoplasmic reticulum; GFP, green fluorescent protein; LS, Linsmaier and Skoog; MT, microtubule; PM, plasma membrane; PBP, pre-prophase band; PSV, protein storage vacuole; TVM, tubular structure of vacuolar membrane; VM, vacuolar membrane.

In higher plant cells, vacuoles show considerable diversity in their shapes and functions. The roles of vacuoles in the storage, osmoregulation, digestion and secretory pathway are well established; however, their functions in cell morphogenesis and cell division are still unclear. To observe the dynamic changes of vacuoles in living plant cells, we attempted to visualize the vacuolar membrane (VM) by pulse-labeling tobacco BY-2 cells with a styryl fluorescent dye, FM4-64. By time-sequence observations using confocal laser scanning microscopy (CLSM), we could follow the dynamics of vacuolar structures throughout the cell cycle in living higher plant cells. We also confirmed the dynamic changes of VM structures by the observation using transgenic BY-2 cells expressing GFP-AtVam3p fusion protein (BY-GV). Furthermore, by using transgenic BY-2 cells that stably express a GFP-tubulin fusion protein [BY-GT16, Kumagai et al. (2001) Plant Cell Physiol. 42: 723], we could study the relationship between the dynamics of vacuoles and microtubules. From these observations, we identified, for the first time, some remarkable events: (1) at the late G1 phase, tubular structures of the vacuolar membrane developed in the central region of the cell, probably in the premitotic cytoplasmic band (phragmosome), surrounding the mitotic apparatus; (2) from anaphase to telophase, these tubular structures invaded the region of the phragmosome, with which the cell plate was being formed; (3) at the early G2 phase, some of the tubular structures expanded rapidly between the cell plate and daughter nuclei, and subsequently developed into large vacuoles at interphase.

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(2000) derived from the ER. From the studies of propeptides of vacuolar proteins, three different types of vacuolar sorting signals have been identified (Neuhaus and Rogers 1998, Matsuoka and Neuhaus 1999).

Plant cell vacuoles often occupy more than 90% of the cell volume in mature tissue cells. Thus, their space-filling properties and solutes play an important role in the growth and morphogenesis of higher plants. During rapid growth of higher

Fig. 1 Staining of vacuolar membranes (VMs) in tobacco BY-2 cells. BY-2 cells were treated with 32 μM FM4-64 for 2 min, then washed with culture medium, and subsequently observed for 1 min to 24 h. First, the plasma membranes (PMs) were immediately stained (1 min). Next, the endosome-like vesicles were also stained with the PMs (5 min to 2 h). The fluorescence of FM4-64 was then translocated from the PMs and vesicles to the vacuolar membranes (VMs) (3–5 h). The fluorescence then became visible only on the VMs (12 h). Some particles appeared in the vacuoles, and could be observed with the VMs (24 h). Bar represents 10 μm.

Fig. 2 Localization of FM4-64 and BCECF fluorescence. BY-2 cells, treated with BCECF (A-1–C-1) and FM4-64 (A-2–C-2), were observed at interphase (A) and metaphase (B), as well as isolated vacuoles (C). In all cases, the green BCECF fluorescence of vacuoles was encircled by the red FM4-64 fluorescence of VMs, as shown in the merged figures (A-3–C-3). Bar represents 10 μm. N represents the nucleus.
Fig. 3  Vacuolar dynamics during the cell cycle in BY-2 cells. BY-2 cells were stained with FM4-64, and were then sub-cultured for over 10 h. Subsequently, vacuolar organization was observed during the cell cycle. At the G1 phase, the well-developed vacuoles could be seen as spaces encircled by VMs. Vacuoles were not observed between the nucleus and the unvisualized PMs. The cytoplasmic strands were also identified as belts running between the VMs. Therefore, the following organization of vacuoles was observed in the midplane optical-sections obtained by CLSM: At the S phase, the vacuoles were compartmentalized by the well-developed cytoplasmic strands, which radiated from the nucleus located at the center of the cell. At G2 phase, the cytoplasmic strands gradually accumulated at the central region of the cell, with the result that the vacuoles became enlarged at both ends and constricted in the central region. At the M phase, many tubular structures of VMs (TVMs, arrowheads) appeared and developed between the nucleus and the PMs, probably in the phragmosome, and they then became to enclose the mitotic apparatus at anaphase. Four large vacuoles were observed at metaphase, and finally two large vacuoles were seen on each side of the cell at telophase, when the TVMs were observed in the region of the phragmoplast. Upper and lower brackets indicate the cytoplasmic band (phragmoplast). Bar represents 10 μm. N represents the nucleus.

Fig. 4  Time-sequence observations of vacuolar structures throughout M phase. In a living BY-2 cell, the dynamic changes of vacuolar structures were time-sequence observed at late G2 phase (0 min), prophase (15 min), prometaphase (30 min), metaphase (45 min), early anaphase (60 min), late anaphase (75 min), early telophase (90 min), late telophase (105 min) and at cytokinesis (120 min). The accumulation of the cytoplasmic strands and the organization of TVMs were continuously observed from the G2 phase to metaphase. Subsequently, the TVMs were also observed around the phragmoplast and cell plate from anaphase to cytokinesis. Bar represents 10 μm.
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Vacuoles have long been known, since their discovery with the light microscope, and were defined as the cell space empty of cytoplasm. More recently, detailed structural analyses have been performed mainly using the electron microscopy. On the other hand, the morphological dynamics of vacuoles has been studied by light microscopy, fluorescence microscopy and confocal laser scanning microscopy (CLSM). In particular, the understanding of the dynamics of vacuolar structures has been facilitated by labeling techniques using endogenous pigments (Palevitz et al. 1981), vital staining dyes (Hillmer et al. 1989, Lazzaro and Thomson 1996, Emans et al. 2002) and GFP (green fluorescent protein)-fusion proteins (Saito et al. 2002, Uemura et al. 2002). To date, however, little is known about the dynamic changes in vacuoles during the cell cycle, primarily because of the lack of appropriate experimental systems in living plant cells.

In this study, therefore, we attempted to observe and follow the dynamics of vacuoles in living tobacco BY-2 cells, which possess well-developed vacuoles and which can be highly synchronized with certain drug treatments. We have employed FM4-64, an amphiphilic styryl dye, for vital staining of the vacuolar membrane (VM). Such styryl FM dyes have been used as markers of endocytosis in animal, fungi and higher plant cells (Betz et al. 1992, Fischer-Parton et al. 2000, Carroll et al. 1998), and for visualizing VMs in yeast and plant cells (Vida and Emr 1995, Emans et al. 2002). We therefore pulse-labeled the BY-2 cells with FM4-64 and observed vacuolar structures with fluorescence microscopy or CLSM. From these observations, including time-sequence ones, we could follow the dynamic changes in vacuoles during cell cycle progression. Furthermore, we could observe these phenomena also in the transgenic BY-2 cells expressing a fusion protein of GFP and AtVam3p, a VM protein of Arabidopsis thaliana (Uemura et al. 2002). As an interesting outcome of these studies, we also identified the development of tubular structures of the VM around the mitotic apparatus, during cell cycle progression from the G2 phase to M phase. This observation is discussed in relation to the origin of interphase vacuoles and the dynamics of cytoplasmic microtubules (MTs) throughout the M phase.

**Results**

**Observation of vacuoles in living BY-2 cells using FM4-64**

To visualize the dynamics of vacuolar structures, the VM of BY-2 cells were stained with FM4-64, a dye that has often been used for staining VMs of living cells of yeast and higher plants (Vida and Emr 1995, Kim et al. 2001). Generally, when the dye is added to the medium, it is first taken up by the...
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...plasma membrane (PM), and then by the endosome membrane, and finally, by the VM. In the VM, the dye emits a red fluorescence by which the VM can be visualized. VM visualization is maintained for some considerable time, so the dynamics of VM can be followed in living cells. By establishing the optimal conditions for staining the VM of living BY-2 cells with FM4-64, we could follow fluorescence localization immediately after labeling BY-2 cells with the dye (Fig. 1). Hence, the PM was identified by red fluorescence at 1 min after the pulse-label of 32 μM FM4-64, whereas endosome-like vesicles were observed from 5 min to 3 h after labeling. At 5 h after labeling, the red fluorescence of the VM began to be observed and was rather stronger than that of the PM. Finally, the fluorescence could be observed only at the VM between 10 and 20 h after labeling. For periods over 20 h after labeling, however, some particles were also stained in the vacuoles (Fig. 1, 24 h). To ensure that FM4-64 actually stained the VM, the BY-2 cells were simultaneously stained with BCECF, a dye that stains the inside of vacuoles (Matsuoka et al. 1997, Mitsuhashi et al. 2000). The green fluorescence of BCECF was indeed found to be encircled by the red fluorescence of FM4-64 (Fig. 2A, B), and was similarly observed in isolated vacuoles (Fig. 2C). We therefore conclude that the fluorescence of FM4-64 demarcates the localization of the VM. Based on these observations, we employed BY-2 cells between 10 and 20 h after labeling with FM4-64 as materials for observing VM dynamics. It should be noted that labeling with FM4-64 did not show any obvious effects on cell division or cell cycle progression of the BY-2 cells.

Vacuolar dynamics of BY-2 cells during cell cycle progression

The structures of the VM were investigated by the labeling technique described above, at each stage of the cell cycle, using highly synchronized BY-2 cells (Fig. 3). At the G1 phase, the vacuoles were identified as compartments enclosed by the VM, with cytoplasmic strands possibly running between them. The vacuoles occupied the major part of each cell, but they were never found between the PM and the nucleus, which was located in the peripheral region of the cell. At the S phase, the vacuoles became compartmented by the cytoplasmic strands that radiated from the central nucleus. At the G2 phase, the cytoplasmic strands gradually accumulated near the central region of the cell, and could fuse together to form the cytoplasmic plate (phragmosome).

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on both sides of the spindle region, and instead many new tubular structures of the VM appeared to encircle the mitotic spindle. From anaphase to cytokinesis, the structures gathered around the mitotic apparatus and finally invaginated into the region where the cell plate was forming. These tubular structures of the VM (TVM) were identified for the first time in this study. Each TVM was several micrometers in diameter and 5–20 μm in length, which was estimated by the optical sections of CLSM (data not shown). When a living BY-2 cell was observed by time-sequence observations, the dynamics of VM structures could be followed in detail throughout the M phase (Fig. 4). The cytoplasmic strands gradually accumulated at the central region of the cell and formed TVMs from late G2 phase to metaphase (Fig. 4, 0–45 min), and they surrounded the mitotic apparatus and then invaded the equatorial region so that they co-localized with the MTs of the developing phragmoplast (Fig. 4, 45–105 min). Consequently, at telophase, the mitotic apparatus surrounded by the TVMs was sandwiched between two large vacuoles (Fig. 4, 105–120 min).

To confirm that the dynamic changes of VM structures are not affected by FM4-64 and that the localization of FM4-64 is restricted to the VMs throughout the cell cycle, we established a BY-2 cell line stably expressing GFP-AtVam3p fusion protein (BY-GV). The organizational changes of VM structures, visualized with GFP fluorescence, were observed also in BY-GV cells as observed with FM4-64 in BY-2 cells throughout the cell cycle (Fig. 5A–C). Furthermore, the images of GFP-AtVam3p were in agreement with those of FM4-64 in the same BY-GV cells (Fig. 5D–F).

**Relationship between the VM and MT structures during cell cycle**

As described above, the VM structures showed dynamic changes during cell cycle progression in BY-2 cells. Similarly, MT structures are known to show changes in spatial organization during the cell cycle (Lambert and Lloyd 1994, Kumagai and Hasezawa 2001). Recently, we established a BY-2 cell line, stably expressing GFP-tubulin (BY-GT16), with which we could follow the dynamics of MT structures during the cell cycle (Kumagai et al. 2001). The relationship between the MT and VM structures was examined by labeling the BY-GT16 cells with FM4-64 (Fig. 6). The MTs were observed only in the cortex at the G1 phase, but also in the cytoplasmic strands at the S phase. At the late G2 phase, the MTs were observed mainly in the preprophase band (PPB) and the thick cytoplasmic strands which were accumulating at the central region of the cell. From time-sequence observations between metaphase and telophase, the TVMs were observed to first enclose the mitotic apparatus of the MTs and then to invade the equatorial region so that they co-localized with the MTs of the developing phragmoplast within which the cell plate was forming. Finally, the TVMs were no longer observed in the region of the collapsing phragmoplast at the cell periphery. Similarly, by time-sequence observations between cytokinesis and early G1 phase, when the cortical MTs were being reorganized from the perinuclear region, new vacuoles could be seen to develop between the cell plate and the daughter nuclei, which were moving away from the cell plate (arrows and arrowheads). They might be organized by fusion of TVMs.

To obtain direct evidence for the relationship between the MT structures and the TVMs, the MTs were destroyed by propyzamide treatment at the G1 phase of synchronized BY-GT16 cells. At 10 h after the release from aphidicolin (at 5 h after the addition of propyzamide), when most of the cells were at the M phase, the TVMs were not arranged so as to encircle the chromosomal region (Fig. 7). Instead, the TVMs were scattered and were co-localized in the chromosomal region. This suggests that the MT structures, the spindle and mitotic apparatus, may be indispensable for the development of the caged structures of TVMs.

**Discussion**

In higher plants the morphogenesis of each tissue depends on the growth and division of each cell. If simplified from the viewpoint of physical architecture, each cell consists of a cell wall, cytoplasm and vacuoles as outer, middle and inner compartments, respectively. In general, plant cells grow by the
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development of these three compartments in interphase until cell division occurs. On the other hand, in the case of rapid growth, such as in stem elongation, the stem cells could elongate by rapid increases in vacuolar volumes without the need for the biosynthesis of cytoplasm which requires considerable energy. Therefore, morphological changes of vacuoles play a primary role in the morphogenesis of cells, tissues and whole plants. Changes in vacuolar volumes also play an important role in the reversible regulation of cell shape, for example, regulation of stomatal opening and closure by changes in vacuolar volume of guard cells (Ward and Schroeder 1994) and leaf movement by those of pulvinus motor cells (Campbell and Garber 1980). To date, vacuoles have been mainly investigated from the viewpoint of the secretory pathway, and many studies have focused on this area (Sanderfoot and Raikhel 1999). However, very few studies have been reported regarding vacuolar morphogenesis in higher plant cells.

In this study, the dynamics of plant vacuoles were examined by a staining method using FM4-64, a dye for visualizing VMs, in tobacco BY-2 cells during cell cycle progression. By this method, changes in vacuolar structures, including the mechanisms of construction and compartmentalization of vacuoles, could be investigated. To gain insights into these phenomena, the use of BY-2 cells is invaluable, because they can be highly synchronized by drug treatments (Hasezawa and Nagata 1991, Nagata et al. 1992). Many cytological, biochemical and molecular-biological studies have been performed using BY-2 cells (Nagata and Kumagai 1999, Kumagai and Hasezawa 2001). In this study, the optimal conditions were established for following the dynamics of vacuolar structures through the cell cycle using FM4-64. The time course of transportation of FM4-64 from the medium to the VMs (Fig. 1) was almost similar to the cases of the protoplasts of the Arabidopsis cultured cells labeled with FM4-64 (Ueda et al. 2001) and of BY-2 cells labeled with FM1-43 (Emans et al. 2002). As a result of observations of optical sections obtained by CLSM, remarkable features of vacuolar compartmentalization were observed that were characteristic of the stages of the cell cycle. In interphase, the vacuoles appeared to be divided into many compartments. At the late G2 phase, the cytoplasmic strands accumulated around the central region of the cell and most of the vacuoles appeared to become contracted. Then, in each optical section of CLSM, at metaphase only four major vacuoles could be observed, and finally at telophase two large vacuoles occupied either side of each cell. Subsequently, at the early G1 phase, some vacuoles originated and developed between the cell plate and the daughter nuclei. These vacuoles were thought to grow and subsequently become the vacuolar compartments observed in interphase.

Interestingly, the tubular structures of VM (TVM) appeared and surrounded the mitotic apparatus throughout M phase. These structures were found for the first time in this study. The TVMs appeared to develop in the perinuclear cytoplasm (phragmosome) at the premitotic stage, then to spread and gather around the mitotic apparatus, and finally to invaginate into the phragmoplast region. Therefore, TVMs might play roles in the support of the mitotic apparatus and/or cell plate formation. In addition, some TVMs might develop and become the vacuolar compartments observed in interphase, as mentioned above. On the other hand, AtVam3p/SYP22, which belongs to the syntaxin family of A. thaliana, has been reported to localize to the VM (Sato et al. 1997). Recently, transgenic A. thaliana lines expressing GFP-AtVam3p fusion protein were established and the localization of the GFP fluorescence to the VM was demonstrated in the transgenic cells (Uemura et al. 2002). In this study, as in the transgenic A. thaliana lines, the VMs in BY-GV cells were visualized by GFP fluorescence. Using the BY-GV cells, we investigated the structures and dynamics of the VMs and could identify all VM structures including TVMs, as observed in the BY-2 cells stained with FM4-64. Furthermore, we could observe that the FM4-64 fluorescence was completely overlapped with that of GFP-AtVam3p in the BY-GV cells stained with FM4-64, as shown in Fig. 5.

The cytoskeleton of higher plant cells is known to show organizational changes during cell cycle progression (Lambert and Lloyd 1994, Kumagai and Hasezawa 2001). Especially around the M phase, the MT structures – the PPB, spindle and phragmoplast – appear and collapse in turns. To examine the relationship between the MT and VM structures, we employed BY-GT16 cells stained with FM4-64. Recently, we established transgenic BY-2 cells stably expressing a GFP-tubulin fusion protein (Kumagai et al. 2001). These BY-GT16 cells could be synchronized by drug treatments, as in the case of original BY-2 cells. Therefore, by time-sequence observations, we could concomitantly follow the changes in MT organizations and VM structures in living plant cells throughout the cell cycle. From these observations, it was found that the MTs run in the cytoplasmic strands sandwiched between vacuoles in interphase, and also that the mitotic apparatus of MTs was surrounded by the VM. Furthermore, from experiments with propyzamide, the MTs were found to be indispensable to the organization of TVMs that encircle the chromosomal region.

In conclusion, we consider that the whole process involved in the dynamic changes of VM structures has been clarified, and that the novel structure of the VM has been identified for the first time by the two different ways. Therefore, the organizations of VM structures observed in this study have been proved to actually appear and disappear during the cell cycle progression. However, the mechanisms of organization and arrangement of the VM structures, which may involve the cytoskeleton, still remain unresolved. For such studies, we are planning to establish a cell line that expresses both of GFP-tubulin and RFP-AtVam3p fusion proteins. By use of such a cell line, we hope to observe the VM structures in great detail and at any time.
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Materials and Methods

Plant material and synchronization

At weekly intervals, suspension cultures of a tobacco BY-2 cell line, derived from seedlings of Nicotiana tabacum L. cv. Bright Yellow 2, were diluted 95-fold with a modified Linsmaier and Skoog (LS) medium (Linsmaier and Skoog 1965), as described by Nagata et al. (1992). The cell suspension was agitated on a rotary shaker at 130 rpm at 27°C in the dark. For synchronization of the cell cycle, 10 ml of 7-day-old BY-2 cells were transferred to 95 ml of the fresh medium and cultured for 24 h with 5 mg liter⁻¹ aphidicolin (Sigma Chemical Co., St. Louis, MO, U.S.A.). The cells were washed with 10 volumes of the medium and then resuspended in fresh medium. The peak mitotic index of ca. 70% could be observed at 9–9.5 h after release from aphidicolin. A highly synchronized cell population starting from the M phase was obtained after sequential treatment with aphidicolin and propyzamide (Kakimoto and Shibaoka 1988, Hasezawa and Nagata 1991, Nagata and Kumagai 1999).

A BY-2 cell line, stably expressing a GFP-tubulin fusion protein, was previously established and designated as BY-GT16 (Kumagai et al. 2001). This cell line could be maintained by almost the same procedures as used for the original BY-2 cell line. In living BY-GT16 cells, the GFP fluorescence, which was localized to MTs, was clearly observable.

Staining of vacuolar membrane and vacuole

To visualize the VM (tonoplast) in BY-2 cells, N-(3-triethy lammoniumpropyl)4-(6-(4-(diethy lamino)phenyl)hexatrienyl)pyridinium dibromide (FM4-64; Molecular Probes Inc., Eugene, OR, U.S.A.) was diluted to 32 μM from a stock solution in DMSO and added to the cell suspension. The cells were incubated for 2 min, washed with fresh culture medium, and then monitored using a fluorescence microscope (BX, Olympus Co. Ltd., Tokyo, Japan) equipped with a digital camera system (DP50; Olympus). To stain the inside of vacuoles, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR, U.S.A.) was used according to Matsuoka et al. (1997). The procedure of isolating vacuoles from the stained protoplasts was according to Sonobe (1990).

Time-sequence observations

For time-sequence observations, 2-day-old or synchronized BY-2 cells, stained with FM4-64, were transferred into 135 mm Petri dishes with 14 mm coverslip windows at the bottom (Matsunami Glass Ind., Ltd., Osaka, Japan). The dishes were placed onto the inverted platform of a fluorescence microscope (IX; Olympus), equipped with a confocal laser scanning head and control systems (CLSM GB-200; Olympus). This observation system, which allows the observation of VM dynamics in BY-2 cells with minimal damage, was found to be ideal for our studies. Moreover, by using BY-GT16 cells, the MT and VM dynamics could be followed almost simultaneously. The cell images were processed digitally using Photoshop software (Adobe Systems Inc., San Jose, CA, U.S.A.).

Transformation and establishment of the BY-2 cell line expressing the GFP-AtVam3p fusion protein

Agrobacterium tumefaciens strain C58C1 was transformed with GFP(S65T)-AtVam3p vector, which was kindly supplied by Dr. M.H. Sato of Kyoto University. A 4-ml aliquot of 3-day-old BY-2 cells was inoculated with 100 μl of an overnight culture of the transformed A. tumefaciens as described by An (1985). After a 2-d incubation at 27°C, the cells were washed four times in 5 ml of LS medium, and were plated onto solid LS medium containing 500 mg liter⁻¹ carbenicillin and 250 mg liter⁻¹ kanamycin. Calluses, which appeared after 10 d, were transferred onto new plates and were cultured independently. The independent calluses of about 1 cm in diameter were transferred to 20 ml in liquid LS medium in 100-ml Erlenmeyer flasks and agitated on a rotary shaker at 130 rpm at 27°C in the dark. After 1 month, a cell line suitable for vacuole observation was selected by identifying GFP-fluorescent cells by the fluorescence microscopy. The cell line, designated as BY-GV (BY-2 cells expressing GFP-AtVam3p fusion protein), could be maintained by 95-fold dilution at weekly intervals as in the case of original BY-2 cells.

Inhibitor treatment

At 5 h after the release from aphidicolin, the BY-GT16 cells were treated with 3 μM propyzamide and were then observed 5 h later by fluorescence microscopy and CLSM.

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