Three-Dimensional Reconstruction of Tubular Structure of Vacuolar Membrane Throughout Mitosis in Living Tobacco Cells

Natsumaro Kutsuna 1, Fumi Kumagai 1, Masa H. Sato 2 and Seiichiro Hasezawa 1, 3

1 Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, 277-8562 Japan
2 Graduate School of Human and Environmental Studies, Kyoto University, Sakyo-ku, Kyoto, 606-8501 Japan

Plant vacuoles are the largest of organelles, performing various functions in cellular metabolism, morphogenesis and cell division. Dynamic changes in vacuoles during mitosis were studied by monitoring tubular structure of vacuolar membrane (TVM) in living transgenic tobacco BY-2 cells stably expressing a GFP-AtVam3p fusion protein (BY-GV). Comprehensive images of the complicated TVM configurations were obtained by reconstructing three-dimensional (3-D) surface structures from sequential confocal sections, using newly developed software, SSR (stereo-structure reconstructor). Using the surface modeling technique, we succeeded for the first time in clarifying the development process of TVMs and the topological relationship between TVMs and large vacuoles. TVMs, initially organized from large vacuoles, elongated to encircle the spindle at metaphase. Subsequently, the TVMs invaded the equatorial region from anaphase to telophase, and then they were divided to the two daughter cells by the cell plate at cytokinesis. When the daughter nuclei were separating from the cell plate, some TVMs enlarged to form large vacuoles near the division site. Spatial analysis revealed that from anaphase until cytokinesis, TVMs connected the two large vacuoles and functioned as a route for inter-vacuolar transport. Furthermore, the experiments using the inhibitor for actin microfilaments indicated that the microfilaments were indispensable for the development and the maintenance of TVMs.

Keywords: 3-D reconstruction — AtVam3p — GFP — Mitosis — Tobacco BY-2 cells — Vacuole.

Abbreviations: 3-D, three-dimensional; BA, bistheonellide A; BY-GV, BY-2 cell line expressing GFP-AtVam3p fusion protein; CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein; MF, microfilament; MT, microtubule; SSR, stereo-structure reconstructor; TVM, tubular structure of vacuolar membrane; VM, vacuolar membrane.

Introduction

Vacuoles constitute the largest organelles in plant cells, and serve as multifunctional compartments (Wink 1993, Marty 1999). The large central vacuole occupies a considerable part of the intracellular volume of most higher plant cells, and over 90% of the cell volume in mature tissues. The space-filling properties and solutes of vacuoles therefore play an important role in the growth and morphogenesis of higher plants. During the rapid growth of higher plants, for example, increased vacuolar volumes can promote cell expansion without the need for cell division. On the other hand, the large size of vacuoles affects the dynamic and physical characteristics of plant cells, and their structural behavior is thought to be important for cell division and morphogenesis.

The morphological dynamics of vacuoles have been studied by light microscopy, fluorescence microscopy and confocal laser scanning microscopy (CLSM), and a greater understanding of their structural dynamics has been facilitated by labelling techniques using endogenous pigments (Palevitz and O’Kane 1981, Palevitz et al. 1981), vital staining dyes (Hillmer et al. 1989, Lazzaro and Thomson 1996, Emans et al. 2002) and green fluorescent protein (GFP)-fusion proteins (Di Sansebastiano et al. 1998, Cutler et al. 2000, Mitsuhashi et al. 2000, Hawes et al. 2001, Darnowski and Vodkin 2002, Saito et al. 2002, Uemura et al. 2002). To date, however, little progress has been made in characterizing the dynamic changes in vacuoles during the cell cycle, primarily because of the lack of appropriate experimental systems in living plant cells.

In order to follow vacuolar dynamics during cell cycle progression, we initially performed vital staining of the vacuolar membrane (VM, tonoplast) of tobacco BY-2 cells harboring developed vacuoles, by pulse-labelling with the styryl dye, FM4-64 (Kutsuna and Hasezawa 2002). At interphase, the vacuoles were compartmentalized by cytoplasmic strands and, by late G2 phase, the cytoplasmic strands, which extended from the central nucleus, gathered to the mid-region of cell where they formed the cytoplasmic plate that supports the mitotic apparatus (phragmosome). Interestingly, the characteristic VM structures were found to surround the mitotic apparatus at M phase, and were recognized as small ellipses on each optical section. However, by observing serial optical-sections, it became evident that the VM structures were tubular in form, and were thus designated as TVM (tubular structure of vacuolar membrane) (Kutsuna and Hasezawa 2002).

The use of FM4-64 for staining VMs is well suited for confirming the existence of thin structures, such as TVM or...
cytoplasmic strands, but is unsuitable for time-sequential or multi-focal observations due to fluorescence fading; thus making analysis of the complex structures and dynamics of TVMs extremely difficult. In this study, therefore, we intended to clarify the structure and dynamics of TVM in order to elucidate the functions of VM structures in plant cell division. We therefore established a BY-2 cell line stably expressing a fusion protein of GFP with AtVam3p/SYP22 (Kutsuna and Hasezawa 2002), which belongs to the syntaxin family of Arabidopsis thaliana proteins (Sato et al. 1997). Using BY-GV cells, we first followed the detailed changes in the vacuolar structures throughout mitosis. We subsequently developed software, termed SSR (stereo-structure reconstructor), to construct three-dimensional (3-D) images of the VM structures, especially TVMs, from each series of optical sections generated by CLSM. From the data obtained with this system, the structure and function of TVMs during mitosis could be analyzed. Additionally, visible effects of actin microfilament (MF) disruption on the structures of vacuoles were demonstrated stereologically. The double-labeling experiments using GFP-AtVam3p and rhodamine-phalloidin suggested that the MFs participated in the structural adjustment of TVMs.

Results

Transgenic BY-2 cells for observing vacuolar structures

Tobacco BY-2 cells were transformed with Agrobacterium tumefaciens, harboring a 35S-GFP-AtVam3p construct, essentially as described by An (1985), and the transformed cell lines were designated BY-GV (BY-2 cells stably expressing GFP-AtVam3p) (Kutsuna and Hasezawa 2002). From several BY-GV cell lines we have chosen a line, designated BY-GV7, for the following observations. The line displayed bright GFP-fluorescence, and it could be maintained by 95-fold dilutions at weekly intervals as in the case for the original BY-2 cells. In 2-day-old BY-GV7 cells, 10–20% of cells were observed in the M phase. Superficially, the shapes and sizes of the BY-2 cells and BY-GV7 cells were indistinguishable. However, the BY-GV7 cells emitted bright GFP-fluorescence that highlighted the VM structures, especially TVM gross morphology. In BY-GV7 cells, the VMs were more clearly labelled with GFP-fluorescence than FM4-64 shown in Fig. 1 and 4 of Kutsuna and Hasezawa (2002). Throughout the cell cycle, the VM structures observed in BY-GV7, including TVMs, were very similar to those observed in two other BY-GV lines whose GFP-fluorescence was slightly lower than that of BY-GV7 (data not shown). The VM structures of BY-GV7 cells were also similar to those of original BY-2 cells stained with FM4-64 (Fig. 1A), and in BY-GV7 cells the GFP-fluorescence perfectly overlapped with the FM4-64 fluorescence (Fig. 1C). The vacuolar morphology could be observed by staining the vacuolar lumen with Alexa 568 hydrazide (Fig. 1B) or 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM; Kutsuna and Hasezawa 2002), and the GFP-fluorescence encircled on the vacuoles was shown by Alexa dye (Fig. 1D). These observations indicated that the fluorescence of GFP-AtVam3p certainly localized on the VMs in BY-GV7 cells. Furthermore, based on the immunoblotting analyses by the antibody against AtVam3p, the amount of the GFP-AtVam3p fusion protein in BY-GV7 cells was not so high (data now shown). From these results, we concluded that the fused protein employed here was incorporated and located into VMs and the vacuolar morphology, including TVMs, was not artificially affected in BY-GV7 cells.

Vacuolar dynamics throughout mitosis

As described above, the BY-GV7 cells showed quite comparable growth rates to the original BY-2 cells, and they could be highly synchronized by aphidicolin. More importantly, their emitted GFP-fluorescence faithfully reflected the dynamic changes in VM structures from G2 phase to G1 phase. As the BY-GV7 observation system was superior, in terms of VM fluorescent brightness, slowness of fading and recovery from fading, to that using FM4-64 (Kutsuna and Hasezawa 2002), the
system was considered suitable for time-sequential or multifocal observations. We therefore aimed to follow the detailed changes in fluorescence during mitosis by CLSM, with special emphasis on TVM dynamics around the mitotic apparatus (Fig. 2). The observations were performed with one randomly chosen cell at 20-min intervals. Thick cytoplasmic strands began to gather near the central region of the cell at late G2 phase (Fig. 2, 20 min, arrowheads). At this time, in optical sections, some vacuolar compartments appeared sandwiched by the cytoplasmic strands and isolated from the large vacuole. From there, the TVMs seemed to develop at the G2/M interface, to surround the mitotic apparatus at metaphase (Fig. 2, 80 min, arrowheads), and then to invade the equatorial region from anaphase to telophase (Fig. 2, 180 min, arrowheads). The TVMs tended to elongate along the longitudinal axis of cell extension, and were then divided by extensive growth of the cell plate at cytokinesis (Fig. 2, 200 min, arrowheads). After cytokinesis, some TVMs that were located between the cell plate and daughter nuclei (200 min, arrowheads) developed into large vacuoles (220 min, arrowheads) at the separating of the nuclei from the cell plate. n, nucleus. Bar = 10 µm.

**Fig. 2** Time-sequence observations of vacuolar structures throughout mitosis. In a living BY-GV7 cell, the dynamic changes in vacuolar structures were time-sequentially observed at the late G2 phase (0–40 min), prophase (60 min), metaphase (100 min), anaphase (160 min), telophase (180 min) and early G1 phase (220 min). At the late G2 phase, thick cytoplasmic strands gathered at the central region of the cell. In a single optical section of the central region, several vacuolar compartments appeared to be isolated from the large vacuole by cytoplasmic strands (20 min, arrowheads). They then changed their forms as TVMs at the G2/M interface (80 min, arrowheads). From anaphase to telophase, TVMs were also observed in the equatorial region (180 min, arrowheads), and tended to align in the longitudinal direction. At cytokinesis (200 min), the TVMs divided into the two daughter cells because of centrifugal expansion of the cell plate developing within the phragmoplast. After completion of cytokinesis, some TVMs between the cell plate and daughter nuclei (200 min, arrowheads) developed into large vacuoles (220 min, arrowheads) at the separating of the nuclei from the cell plate. n, nucleus. Bar = 10 µm.

---

3-D reconstruction of vacuolar structures

To understand the spatial configuration of TVMs and their relationship with large vacuoles, we performed 3-D reconstructions of the vacuolar structures from a series of confocal images taken along the z-axis by CLSM. Due to the lack of an appropriate VM processing system, we developed a new software package, SSR (Kutsuna et al., in preparation), to perform 3-D modeling of a series of optical sections from the BY-GV7 cells. To overcome the complexity of vacuolar structures as well as anisotropic resolution, which is typical of serial sections obtained by CLSM, we employed parallel contour reconstruction by which surfaces of 3-D objects are reconstructed by connecting planar contours that represent cross-sections through the objects (Fuchs et al. 1977). The obtained vacuolar structures could be observed from any angle, and measurements of their volume and surface area could be obtained.

The 3-D vacuolar structures, which were reconstructed by SSR at each stage of the cell cycle, are shown in Fig. 3. Each image was constructed from serial optical sections obtained by CLSM at 0.5–1.0 µm intervals. In the image at the late G2 phase, TVMs have not been developed yet, and the cytoplasmic strands run penetrating the large vacuole around central region of the cell (Fig. 3A). Although some vacuolar compartments appeared to be isolated from the large vacuoles on the single optical section (Fig. 2, 20 min), in fact these compartments connected with each other and with the large vacuole...
In the image of vacuolar structures at prophase, the cross-sections of vacuoles are divided into three classes by the area size (Fig. 3B). The large sections, the middle ones and the small ones are shown in blue, sky-blue and green, respectively. This indicated that the TVMs, with small diameters and shown in green, extended from the sky-blue compartments, which were located at the periphery of the nucleus and in the mid-region of the cell (Fig. 3B). The TVMs were few in number and short in length at this stage. In the image obtained at metaphase, the large vacuole was divided in two by the cytoplasm around the mitotic apparatus, proving that the TVMs elongated around the mitotic apparatus and connected the two large vacuoles (Fig. 3C). This finding is the first demonstration of the TVM dynamics using 3-D modeling. In three optical sections, near the metaphase cell surface, obtained by CLSM at 0.7 μm intervals, the large vacuole was bisected by the cytoplasm around the mitotic apparatus (Fig. 4). When the TVM was traced from left to right, from the point where it was connected to the large vacuole (Fig. 4, arrowheads), the TVM appeared to connect both large vacuoles (Fig. 4). When the vacuoles of metaphase BY-2 cells were stained with fluorescent dye, BCECF, large vacuoles with faint TVMs could be observed (Fig. 5A, left). Photobleaching was performed with strong excitation light only in the dashed area, after taking the photograph. After a minute, BCECF fading was observed both in the photobleached vacuole and in an additional vacuole (Fig. 5A, right). This not only suggested that both large vacuoles, which were bisected by the cytoplasmic plate, were topologi-
3-D structure of vacuoles in living BY-GV cells

3-D structure of vacuoles in living BY-GV cells

cally connected by TVMs, but also that the vacuolar contents were always able to diffuse between the large vacuoles through the TVMs.

Role of actin MFs on the morphology of TVMs

To clarify the mechanism for development and maintenance of tubular structures characteristic of TVM, effects of cytoskeleton inhibitors on the morphology of vacuoles were observed using the BY-GV7 cells. We found that development of TVMs were inhibited by the treatment with 1 μM bisthconiellide A (BA), a dimeric macrolide, that was recently reported to inhibit polymerization of G-actin (Saito et al. 1998, Hoshino et al. 2003). At the late G2 phase, BA treatment caused the formation of several spherical vacuoles around the nuclei (Fig. 6A-1, arrowheads). The 3-D image showed that the newly formed spherical vacuoles were not connected to the large vacuoles via VMs, and connectivity between two large vacuoles was disrupted (Fig. 6A-2). Moreover, the disruption of MFs inhibited the development and maintenance of TVMs at the metaphase (Fig. 6B). Instead of TVMs, the spherical vacuoles were observed (Fig. 6B-1). Treatments with 100 μM cytochalasin B and D, other actin inhibitors, caused similar changes in the structure of vacuoles (data not shown).

The disappearance of TVMs, by the destruction of MFs, might cause the loss of luminal connection between the two large vacuoles. Thus, the vacuolar lumen of BY-2 cells was vital-stained with BCECF, and then their MFs were destroyed with BA. Subsequently, the photobleaching was performed on

\[ Fig. 4 \quad \text{Connections between the large vacuoles and TVMs at metaphase of a BY-GV7 cell. Three optical sections were obtained by scanning from the cell surface at 0.7 μm intervals. The large central vacuole was divided into two by the cytoplasm, including the mitotic apparatus, from where the TVMs were derived (arrowheads). The TVMs were observed to connect the two large vacuoles through three serial sections. l, large vacuole. Bar = 10 μm.} \]

\[ Fig. 5 \quad \text{Connectivity of the lumen of two large vacuoles at metaphase. The vacuolar lumens of metaphase BY-2 cells were stained with BCECF, and the cells then observed by fluorescence microscopy. Photobleaching was performed by the irradiation with the strong excitatory light only to the dashed area. (A) The large central vacuole of the central cell was divided into two sections (pre-bleach). In addition to the large vacuoles, TVMs were also identified by faint fluorescence of BCECF (arrowheads). However, after photobleaching, the fading of the green fluorescence was not only limited to the left vacuole but also observed in the right vacuole (post-bleach). (B) A BA-treated cell. Instead of TVMs, some spherical vacuoles were observed around mitotic apparatus (pre-bleach). The fading, observed in the left large vacuole, did not spread to the right vacuole (post-bleach). l, large vacuole. Bars = 10 μm.} \]
the metaphase cells, which were untreated or treated with BA (Fig. 5). In contrast to the untreated cell (Fig. 5A, right), the BCECF fading did not spread to another large vacuole in the BA-treated cell (Fig. 5B, right). As expected from the 3-D image (Fig. 6), the disruption of MFs actually caused the separation of vacuoles and the lumenal disconnection between the two large vacuoles, suggesting that the MFs may be necessary for the maintenance of TVMs as the lumenal connection.

These experiments with the inhibitor indicated that the MFs were involved in the organization of TVMs. To gain insight into the spatial relationship of them, BY-GV7 cells were stained with rhodamine-phalloidin to visualize the MFs and were observed by CLSM (Fig. 7). Some TVMs seemed to be surrounded by or colocalized with the MFs during mitosis (Fig. 7A, B). On the other hand, when pretreated with BA for 30 min, such MFs were rarely observed (Fig. 7C), as described in Hoshino et al. (2003).

Discussion

In this study, we have established a cell line BY-GV7 that stably expresses a GFP-AtVam3p fusion protein, and examined the dynamics and 3-D structures of plant vacuoles during mitosis. GFP fluorescence in the VM of BY-GV7 cells was more intensive and durable than by staining with the fluorescent dye, FM4-64. In the latter case, the fluorescence gradually darkened, and some particulate artifacts appeared in the vacuoles within several days (Kutsuna and Hasezawa 2002). In con-
contrast, the BY-GV7 cells showed stable GFP fluorescence due to the constitutive expression of the GFP-AtVam3p fusion protein by the CaMV 35S promoter. From the observations of BY-GV7 cells double-labelled with GFP-AtVam3p and FM4-64 (Fig. 1C) or GFP-AtVam3p and Alexa hydrazide (Fig. 1D), the fluorescence of GFP-AtVam3p seemed to be located in the whole VMs and not to be located in the limited part of VMs. In this context, it has been previously demonstrated that the GFP-AtVam3p was localized to the VMs and could be used as a fluorescent probe for the VM structures in transgenic A. thaliana (Uemura et al. 2002). Specific features of the BY-GV7 cells, notably fluorescence intensity, durability and sharpness, enabled us to perform not only studies on vacuolar morphology in living cells but also time-sequential and multi-focal observations. In addition, the shapes and sizes of the BY-GV7 cells were indistinguishable from the original BY-2 cells, their growth rates were essentially similar, and the cells could be synchronized by the same method described in Nagata and Kumagai (1999) (data not shown). Therefore, at the peak of mitotic index, a large number of cells at various mitotic and premitotic stages could be easily observed at the same time.

The structure of VMs in living plant cells are dynamically modified within a brief time-span, and their configurations are sometimes very complicated and intricate. Spherical structures, designated as ‘bulbs’, have been reported within the lumen of vacuoles in rapidly expanding cells of A. thaliana (Saito et al. 2002), and cylindrical and sheet-like structures that invaginate into the vacuolar lumen have been observed in several tissues of transgenic A. thaliana expressing a GFP-AtVam3p (Uemura et al. 2002). These structures were reported to exhibit considerable motility. Time-lapse observations and acquisition of 3-D images were indispensable for comprehending these structures. During mitosis, TVMs also displayed dynamic changes in their conformations, with entangled and complicated structures (Fig. 2), and 3-D image processing served as a powerful tool for understanding such structures. Of the numerous approaches to 3-D image processing, we selected parallel contour reconstruction, which performs surface reconstructions, and then developed specific computer software, SSR, as tool for reconstructing 3-D structures and analyzing the series of CLSM images. By employing the contour-based modeling system, we have succeeded in reconstructing and visualizing 3-D vacuolar structures containing TVMs (Fig. 3, 6). Compared to the single optical sections, the 3-D images reconstructed by SSR provided more comprehensive views of the morphological changes induced by the MF disruption (Fig. 6). In addition, the newly developed SSR software could be used to quantify 3-D structures, such as of the surface area and volume of both vacuoles and whole cells (data not shown). It is notable that the SSR software can be widely applied to various biological objects, especially for membranous structures even in large plant cells, in which the CLSM images are prone to anisotropic resolution and to greater topological differences between serial sections of whole cells. In addition, by combining SSR with a real-time observation system using CLSM, we think that we will be able to monitor the dynamic images of 3-D structures over a long time-period. In such cases, SSR may be suitable for investigations of plant cell morphology.

Our results on the dynamics of TVMs are summarized in Fig. 8. At the late G₂ phase, the cytoplasmic strands converge on the central region of the cell, and compartmentalize the large central vacuole around the nucleus (Fig. 8A). The TVMs then elongate from some of these vacuolar compartments at the G₂/M interface. Although the large vacuole is segregated into two parts by the cytoplasm and mitotic apparatus, they are connected to each other by the TVMs at metaphase (Fig. 8B). Some TVMs invade the equatorial area after chromosomal segregation at anaphase. When the cell plate is formed, the TVM bridges between the large vacuoles are cut off by the developing cell plate (Fig. 8C). After cytokinesis, some TVMs between the cell plate and daughter nuclei develop into large vacuoles at the early G₁ phase (Fig. 8D). With respect to TVM behavior, the vacuolar-tubular continuum in the secretory trichomes of chickpea were reported to be continuous between the stalk cells.
and to function in the rapid delivery of solute from the trichome base to the secretory head cells (Lazzaro and Thomson 1996). In BY-GV7 cells, the TVMs connected the two large vacuoles and appeared to be cut by the cell plate during cell plate formation. It is possible that this TVM bridge is the precursor of the continuum observed in stalk cells of chickpea. Conversely, if TVMs accidentally escape being cut off at cytokinesis, the TVMs may penetrate into the new cell wall as in the case of the vascular-tubular continuum.

The viscosity of the lumen of lytic vacuoles is considerably lower than that of the cytoplasm, a property that appears valuable for bulk transport. In fungal hyphae, motile tubular vacuoles have been implicated in longitudinal transport of nitrogen and phosphorus compounds (Ashford 1998). In our photobleaching experiment (Fig. 5A), we demonstrated that lumens of the two large vacuoles were connected by TVMs, and that luminal solutes could probably diffuse freely. Compared to the result of BA treated cells in Fig. 5B, the BCECF fading in Fig. 5A was not due to the cellular damage caused by the excitation light of photobleaching. It is uncertain how solutes are mixed within the lumen and whether any mechanism of positive stirring exists. The time-course pattern of BCECF fading could be explained by passive diffusion. The connectivity of vacuolar lumens through the TVMs also suggests that specific conditions, for example osmotic pressure, pH, membrane potential, and electrolyte concentrations, were maintained at equal levels in the two large vacuoles.

It is noteworthy that TVMs were found connected to the large vacuole throughout mitosis and cytokinesis, suggesting that they may function as branches of the large vacuole. Some TVMs invaded the equatorial region between anaphase and telophase (Fig. 2, 180 min), and subsequently co-localized with the developing phragmoplast within which the cell plate was forming (Kutsuna and Hasezawa 2002). For cell plate formation, large amounts of membranous components appear to be consumed by vesicle transport of the cell plate materials. In the formation (Kutsuna and Hasezawa 2002). For cell plate formation, large amounts of membranous components appear to be consumed by vesicle transport of the cell plate materials. In the

In summary, using our newly developed SSR software as a tool for modeling, analyzing and viewing 3-D biological image data, we have been able to demonstrate the following: (1) BY-GV7 cell line is suitable for time-sequence observations of VMs; (2) SSR can clarify complicated cellular structures such as TVMs; (3) each TVM is a tubular structure and is derived from the periphery of the large vacuole; (4) TVMs actually connect two large vacuoles, and act as a pathway for the transport of soluble vacuolar contents during mitosis; and (5) development and maintenance of TVMs depend on MFs. Our future studies will focus on analyzing in detail the structure and function of plant vacuoles, especially in TVMs, using BY-GV7 cells and our SSR software.

**Materials and Methods**

**Plant material**

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 (Nagata et al. 1992). The cell suspension was agitated on a rotary shaker at 130 rpm at 27°C in the dark.

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

*Agrobacterium tumefaciens* strain, C58C1, was transformed with the GFP(S65T)-AtVam3p construct. 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 (An 1985). After a 2-day incubation at 27°C, the cells were washed four times in 5 ml LS medium, and then plated onto solid LS medium containing 250 mg liter−1 kanamycin and 500 mg liter−1 carbenicillin. Calluses that appeared after 10 d were transferred onto new plates and cultured independently until they reached a diameter of about 1 cm, at which time they were transferred to 20 ml liquid LS medium in 100-ml Erlemeyer flasks and agitated on a rotary shaker at 130 rpm at 27°C in the dark. After 1 month, a cell line suitable for VM structural observations was selected by identifying GFP-fluorescent cells by fluorescence microscopy. The selected cell line, designated BY-GV7 (BY-2 cell line expressing GFP-AVam3p fusion protein No.7), could be maintained by 95-fold dilutions at weekly intervals, and be synchronized similarly to the original BY-2 cells.

**Staining of vacuoles and vacuolar membranes**

To stain the inside of vacuoles, 2,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Molecular Probes Inc., Eugene, OR, U.S.A.) and Alexa 568 hydrazide (Molecular Probes) were used. BCECF-AM was added to the cell suspension at the final concentration of 10 μM. The cells were incubated for 1 h at 27°C, washed with fresh culture medium, and then incubated for an additional 6 h at 27°C. Alexa 568 hydrazide was used essentially according to Emans et al. (2002). To visualize the VM in BY-2 cells, N-(3-triethylaminomunpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64, Molecular Probes) was used (Kutsuna and Hasezawa 2002). Cells between 10 and 20 h after labeling were used to observe VMs.

**Staining of actin MFs**

For simultaneous observations of MFs and VMs, BY-GV7 cells were suspended in a solution containing 50 mM PIPES (pH 6.8), 1 mM MgSO4, 5 mM EGTA, 1% glycerol, 3% sucrose, 0.03% saponin (ICN Biomedicals Inc., Aurora, OH, U.S.A.), and Alexa 568 hydrazide (Molecular Probes) were used. BCECF-AM was added to the cell suspension at the final concentration of 10 μM. The cells were incubated for 1 h at 27°C, washed with fresh culture medium, and then incubated for an additional 6 h at 27°C. Alexa 568 hydrazide was used essentially according to Emans et al. (2002). To visualize the VM in BY-2 cells, N-(3-triethylaminomunpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64, Molecular Probes) was used (Kutsuna and Hasezawa 2002). Cells between 10 and 20 h after labeling were used to observe VMs.

**Photobleaching of BCECF within vacuoles**

The BY-2 cells were stained with BCECF as described above, and monitored with a fluorescence microscope (BX, Olympus) equipped with a CCD camera system (DP70, Olympus). Following observation, and confirmation that BCECF fluorescence was limited to vacuolar lumens, photobleaching was performed by irradiating only a small area of the cell with strong excitation light using an iris.

**Acknowledgments**

This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (No. 10219201 and 15031209) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Nissan Science Foundation (S. H.).

**References**


Hoshino, H., Yoneda, A., Kumagai, F. and Hasezawa, S. (2003) Examining the roles of actin depleted zone (ADZ) and preprophase band (PPB) in determining the division site of higher plant cells using a BY-2 cell line expressing GFP-tubulin (BY-GT). *Protoplasma* in press.


(Received April 18, 2003; Accepted July 30, 2003)