Chapter 5

Plant Cell Vacuoles: Staining and Fluorescent Probes

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Abstract

In plant cells, vacuoles are extremely important for growth and development, and influence important cellular functions as photosynthesis, respiration, and transpiration. Plant cells contain lytic and storage vacuoles, whose size can be different depending on cell type and tissue developmental stage. One of the main roles of vacuoles is to regulate the cell turgor in response to different stimuli. Thus, studying the morphology, dynamics, and physiology of vacuole is fundamentally important to advance knowledge in plant cell biology at large. The availability of fluorescent probes allows marking vacuoles in multiple ways. These may be fast, when using commercially available chemical dyes, or relatively slow, in the case of specific genetically encoded markers based on proteins directed either to the membrane of the vacuole (tonoplast) or to the vacuole lumen. Any of these approaches provides useful information about the morphology and physiology of the vacuole.

Key words Vacuole, Vacuole lumen, Tonoplast, Chemical dyes, Fluorescent-tagged vacuolar proteins

1 Introduction

The plant vacuole is one of the largest subcellular compartments, mainly enriched in water, that in some conditions can occupy up to 90% of the total cell volume [1, 2]. The plant vacuoles exist as storage and lytic compartments [3, 4]. Beside a role as water storage compartment, which is a critical element in cell turgor and cell growth processes, the vacuole is also extremely important to accumulate metabolites [1]. The vacuole is also important for seemingly unrelated processes, including photosynthesis. For example, changes in vacuole morphology and size control the aperture or closure of the stomata by guard cells. This in turn modulates CO_2 intake and O_2 extrusion [5, 6], a process that is critical for photosynthesis. Additionally, in CAM-based photosynthesis, vacuoles store CO_2 that is taken up at night and used during the day for photosynthesis. Thus studying how and when the morphology of the vacuole is regulated can provide important clues to understand mechanisms that directly or indirectly drive important processes

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including cell expansion, cell growth and photosynthesis [1]. Intriguingly, key mechanisms governing vacuole morphology are yet to be discovered and their identification may be facilitated by the use of fluorescent probes which mark this subcellular compartment in several ways. The probes provide a good degree of flexibility to investigators to study the physiology and morphology of the vacuole as well as cargo traffic to the tonoplast or the vacuole lumen.

As follows, we will describe different vacuolar labeling methods, which can be used for different purposes.

1.1 Chemical Dyes

1.1.1 Neutral Red

Neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine) is a lipophilic phenazine dye and is considered a nontoxic compound, at least for plant systems [7]. Root growth and cytoskeleton structures (actin and microtubules) are not affected after short NR staining treatment. It is also a relatively inexpensive chemical. The compound penetrates easily through biological membranes and has been used as a vital stain to label vacuoles in plant cells, but also in other organisms like fungi and the lysosomes of animal cells [7] using light microscopy [8]. In its unprotonated form, NR penetrates the plasma membrane and tonoplast. Once inside the vacuole, the fluorescent dye is protonated and trapped (Fig. 1). Although the dye can be used to stain the vacuole, it has also been described to visualize plant structures (such as protoxylem, metaxylem elements, and Casparian bands) [7]. In confocal microscopy analyses, NR can be excited using a 488, 514, or 543 nm laser line; the maximum fluorescence emission is in the range between 550 and 650 nm.

1.1.2 FM4-64

Another dye that can be used to label the vacuole is the lipophilic styryldye,N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64). This dye binds the lipids of the outer leaflet of the plasma membrane, which consists predominantly of phosphatidylcholine, sphingomyelin, and glycolipids. From the plasma membrane, FM4-64 reaches endosomal compartments and ultimately the vacuole [9] (Fig. 1). Because a delay of the dye in reaching the vacuole indicates plasma membrane to vacuole trafficking defects, FM4-64 has been used in mutagenesis screens based on time-course internalization of the dye to identify trafficking elements in yeast [10]. In confocal microscopy, FM4-64 can be excited using 488, 514, 543, and 561 nm laser lines; the maximum fluorescence emission is in the range between 600 and 650.

1.2 Fluorescent-Tagged Vacuolar Proteins

1.2.1 AFVY-RFP

A useful fluorescent marker for the central vacuole is AFVY-RFP, a soluble marker based on the fusion of a monomeric red fluorescent protein (RFP) to the tetrapeptide AFVY, a C-terminal sorting signal of soluble proteins to the vacuole [11] (Fig. 2) (*see* **Note 1**). The fluorescence can be detected using a 560 nm laser line (excitation); the emitted signal can be detected between 560 and 650 nm.

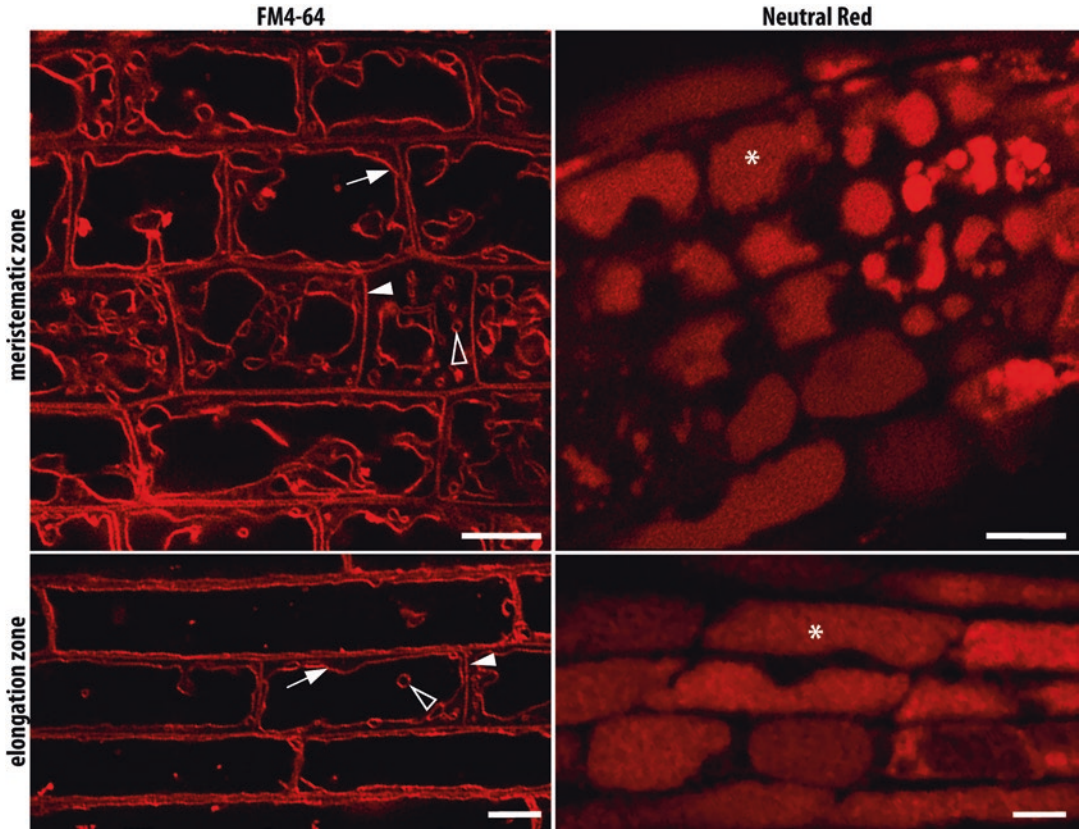

Fig. 1 Staining of vacuoles with fluorescent dyes. (*Left panels*) *A. thaliana* (6 DAG) root seedlings were incubated with FM4-64 (30 min), and observed after 2 h of incubation in ½ LS medium. Arrows indicate tonoplast, empty arrows points to bulbs, and arrowheads point to plasma membrane. Images were acquired with Nikon A1Rsi, using a 514 nm laser as excitation, and fluorescence signal was acquired with filter cube 595/50. (*Right panels*) Confocal images of roots of *A. thaliana* 6 DAG (days after germination) seedlings that were incubated with Neutral Red (15 min) to stain the vacuolar lumen. Asterisk indicates the vacuolar lumen. Imaging was performed with Zeiss LSM510 confocal microscope, using a 543 nm laser as excitation, and fluorescence signal was acquired with filter cube LP560. Bar = 10 μm

1.2.2 VAC-YFP

To mark the tonoplast, various fluorescent-tagged proteins have been used. Here we describe VAC-YFP, a tonoplast marker based on the fusion of the C-terminus of a γ-TIP protein [12] to the yellow fluorescent protein (YFP) (Fig. 2). The fluorescence can be detected using a 488 or 514 nm laser line (excitation) and the emitted signal can be detected between 560 and 600 nm, as previously described [12].

1.3 Additional Fluorescent Vacuolar Markers Available

Among the numerous fluorescent markers used to label the vacuole, two have been used to label either the lytic vacuole (LV), which can be marked by Aleu-GFP [4], or the protein storage vacuole (PSV), which can be labeled using GFP-Chi [13, 14]. Another important marker to mention is δTIP-GFP (tonoplast intrinsic pro-

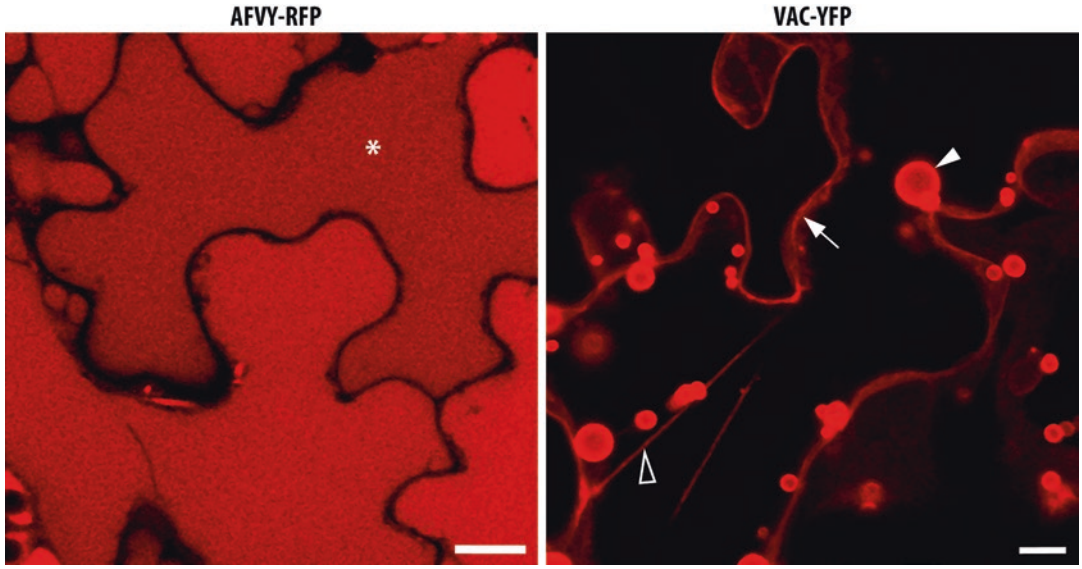

Fig. 2 Staining of vacuoles with fluorescent proteins. (*Left panel*) Confocal images of Arabidopsis abaxial epidermal cells (9 DAG) stably expressing AFVY-RFP, which marks the vacuolar lumen indicated by an asterisk. Imaging was performed with Zeiss LSM510 confocal microscope, using a 543 nm laser as excitation, and fluorescence signal was acquired with LP560 filter. (*Right panel*) VAC-YFP (12 DAG) in abaxial epidermal cells. Arrow points to tonoplast, arrowhead indicates bulbs, and empty arrowhead indicates transvacuolar-strand. A Zeiss LSM510 confocal microscope, with a 514 nm laser as excitation was used, and fluorescence signal was detected with filter cube BP580/40. Bar = 10 μm

tein), a tonoplast fluorescent marker. This marker in the past has been used as tool to screen for vacuolar mutants after ethyl methanesulfonate (EMS) treatment, a chemical inducing nucleotides changes throughout the genome [15]. VAMP711-YFP can also be used to the list of useful markers for tonoplast labelling [16].

2 Materials

2.1 Plant Growth

1. Linsmaier and Skoog medium (LS) plates, with the pH buffered to 5.7 ± 0.2 containing macronutrients, micronutrients, and vitamins. For 1 L of medium (here named 1/2 LS) use 2.35 g of LS, 1% (w/v) sucrose, and 0.8% (w/v) agar, and then autoclave the media for 25 min.

2.2 Preparation of Dye Solution

1. Neutral Red: stock solution of 1 mM in ethanol, keep at −20 °C. Aliquot the stock solution to avoid cycle of thawing and freezing. Protect the dye from light exposure.
2. FM4-64: stock solution of 500 μM in DMSO. Aliquot the stock solution in eppendorf tube an amount equal to 2 μL, then store at −20 °C. Protect the dye from light exposure. Add

500 μL of ½ LS medium to each eppendorf tube containing 2 μL of stock solution to prepare the working solution.

2.3 Stable or Transient Expression of Vacuolar Reporters in Arabidopsis Lines

1. Seeds stratification: LS medium plates. For 1 L of medium (here named 0.25× LS) use 1.18 g of LS, 1% (w/v) sucrose, and 0.8% (w/v) agar, and then autoclave the media 25 min.
2. Cocultivation medium: LS Medium 0.25×, 100 μM acetosyringone, 0.005% Silvet L-77 [17].
3. Wash solution: 10 mM $MgCl_2$ (*see* **Note 2**), 100 μM acetosyringone [17].

2.4 Microscope

Confocal imaging can be performed with a variety of confocal microscopes, including, Zeiss LSM510 META and a Nikon A1Rsi, using a 40×, 60×, or 63× oil objective. Set for each fluorescent probe, the excitation and emission wavelengths as follow:

- For Neutral Red: 543 nm (excitation) and 560–650 nm (emission).
- For FM4-64: 514 nm (excitation) and 600–750 nm (emission).
- For YFP: 514 nm (excitation) and 530–600 nm (emission).
- For RFP: 560 nm (excitation) and 560–650 nm (emission).

3 Methods

3.1 Arabidopsis Root Staining with Neutral Red

1. Stratify *A. thaliana* seeds on ½ LS plate, seal the plate with micropore tape 3M and incubate in a growth chambers from Percival Scientific under standard *A. thaliana* growth conditions (22 °C of temperature, 55% of humidity) in 16 h of light and 8 h of dark cycle.
2. Dispose the plates vertically to image root seedlings or position horizontally to take picture of the abaxial pavemental cells in cotyledon or leaf cells.
3. Use 3–12 DAG Arabidopsis seedlings.
4. Incubate seedlings (a number max of 3 for tube or well) or young leaf (25 mm^2) with 1 μM working solution for 5–15 min (*see* **Note 3**). The seedlings can be incubated directly in an eppendorf tube; if the number of samples to analyze is elevated then a multiwell plate (24 wells) can be used. Add 1 mL of liquid medium for each well used.
5. Add the fluorescent probes to the medium to a final concentration of 1 μm and mix up and down with the pipette.

6. Transfer the seedlings into the eppendorf or multiwell plate containing the medium with the fluorescent probe and incubate for 15–30 min in the dark (*see* **Note 4**).
7. Wash the seedlings by transferring them into a new well supplemented with fresh medium without the probe. Wash the sample at least three times.
8. Place the seedlings on a glass slide and image with a confocal microscope. If you want to image only the root, keep the cotyledons out of the coverslip. In this way you may be able to flatten the sample easily. Vice versa if you want to capture images of cotyledons it possible to cut one cotyledon off the seedling and observe it. Another option is to germinate the seeds directly on plate containing Neutral Red.

3.2 Arabidopsis Root Staining with FM4-64

1. Use 3–9 DAG Arabidopsis seedlings.
2. Incubate the roots (a number max of 3 for tube or well) with 1 μm working solution for 30–45 min (*see* **Note 5**).
3. The seedlings can be incubated directly in an eppendorf tube or a multiwell plate (24 wells) as in Subheading 3.1, **step 2**.
4. Add the fluorescent probes to the medium to a final concentration of 1 μm and mix up and down with the pipette.
5. Transfer the seedlings into the eppendorf tube or well containing the medium with the fluorescent probe and incubate for 30–45 min in the dark (*see* **Note 6**).
6. Wash the seedlings by transferring them into a new well supplemented with fresh medium without the probe. Let the incubation proceed for 1.5–2 h to get a noticeable signal at the tonoplast membrane. Wash the sample at least three times.
7. Place the seedlings on a glass slide and image to a confocal microscope. For sample handling procedures *see* Subheading 3.1, **step 6**.

3.3 AVFY-RFP and VAC-YFP Samples for Confocal Microscopy Analyses

3.3.1 Stable Expression of Genetically Encoded Vacuolar Markers in A. thaliana

1. Sterilize *A. thaliana* seeds expressing the transgene (AFVY-RFP or VAC-YFP), as follow: Pour around 40 seeds in an eppendorf tube, then add 1 mL of ethanol 70%, invert the tube, centrifuge to low speed and discard the solution. Add 1 mL of 20% bleach solution invert the tube few times, centrifuge and discard the liquid by pipette. Wash three times with water then stratify the seeds on plate (LS 0.25× medium) and vernalize at 4 °C for 48 h.
2. Transfer the plates in the growth chamber in a 16 h light and 8 h dark cycle, at 22 °C.
3. Perform confocal microscopy analyses on seedlings starting a 3 days after germination until desired to follow the vacuole marker. Usually, we observe seedlings up to 12 DAG.

3.3.2 Transient Expression of Genetically Encoded Vacuolar Markers in A. thaliana

1. Wild-type *A. thaliana* seeds were sterilized as follow: Pour around 200 seeds in an eppendorf tube, then add 1 mL of ethanol 70%, invert the tube, centrifuge to low speed and discard the solution. Add 1 mL of 20% bleach solution invert the tube few times, centrifuge and discard the liquid by pipette. Wash three times with water then stratify the seeds on plate (LS 0.25× medium) and vernalize at 4 °C for 48 h.
2. Transfer the plates in the growth chamber with 16 h light and 8 h dark, at 22 °C.
3. Two days after germination prepare the cocultivation media. In this way we make sure to use young seedlings with an age of 4–5 days after germination.
4. Three days after germination inoculate the agrobacterium (GV3101) carrying the binary vector with the vacuolar marker of interest, into LB with appropriate antibiotics, shake at 200 rpm, 28 °C until the optical density (OD) read at 600 nm reach a value which is greater than 1.5.
5. Centrifuge the agrobacteria culture at low speed 5000 rpm (2655 × *g*) for 3 min then discard the supernatant.
6. Resuspend the agrobacteria in 10 mL of wash solution by vortex. Repeat the last two steps one more time.
7. Resuspend the agrobacteria pellet in 1 mL of wash solution.
8. Add Agrobacteria to 10 mL of cocultivation media to reach a final optical density of 0.5, then pour the media in the plate.
9. Incubate the plates in the dark for about 24 h.
10. Open the plates remove the media, and wash at least twice with 10 mL of cocultivation media.
11. Seal the plate with micropore 3M and incubate in standard growth conditions.
12. Perform confocal microscopy on cotyledons or root tissue 48–96 h after transformation (*see* **Note 7**).

4 Notes

1. To tag proteins into the vacuole lumen it is suggested to use monomeric RFP because is less sensitive to acidic pH.
2. It is suggested not to autoclave $MgCl_2$, but only sterilize by 0.22 μm filter.
3. The experimental conditions described in this protocol are for young cotyledons (3–12 DAG) and or a young true leaf. It suggested increasing the amount of dye used or the incubation time for older tissues.

4. The incubation time depends on the age of the seedlings: 15 min for 3 DAG root while 30 min for 12 DAG roots.
5. The experimental conditions described in this protocol are for young root seedlings (3–9 DAG). Increasing the amount of dye used or the incubation time will increase the possibility to stain also older tissue.
6. The incubation time depends on the age of the seedlings, 30 min for 3 DAG root while 45 min for 12 DAG seedlings.
7. For transient expression to image a good fluorescent signal into the vacuole or tonoplast it is necessary to observe the sample after 48 h because the signal could be higher in the endoplasmic reticulum before this time.

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