

Qualitative difference between “bulb” membranes and other vacuolar membranes

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Key words: plant growth, SNARE, Rab-GTPase, AtVam3, AtRab75, vacuolar membranes, “bulb”

Abbreviations: SNARE, soluble N-ethyl-maleimide sensitive factor attachment receptor proteins; DAG, day after germination

“Bulb” is a mobile and complex structure appearing in vacuolar membrane of plant cell. We recently reported new fluorescent marker lines for bulbs and bulb-less mutants. We tried multicolor visualization of vacuolar membrane to show distinct segregation of bulb-positive protein (γ TIP or AtVAM3) and bulb-negative protein (AtRab75). Unexpectedly, GFP-AtRab75 resulted to localize in bulb under the condition of co-expression with TagRFP-AtVAM3. The signal intensities of GFP-AtRab75 and TagRFP-AtVAM3 were quantified and compared. The result indicates that TagRFP-AtVAM3 is concentrated in bulb than GFP-AtRab75.

The plant vacuole accomplishes a variety of functions and is essential for plant growth and development. The vacuolar membrane of plant cells has a very complex three-dimensional configuration with dynamic features. We previously identified a “bulb” on the vacuolar membrane of growing *Arabidopsis* cotyledon.^{1,2} A bulb is a complex, mobile structure that forms on the continuous vacuolar membrane (Fig. 1A and B). Our results suggested that γ -TIP (TIP1;1)-GFP molecules were concentrated in bulbs; in contrast, GFP-AtRab75 (AtRABG3b) molecules were segregated (Figs. 1C, 2A and B). These results indicate that qualitative differences between bulbs and other vacuolar membranes (termed the “peripheral vacuolar membrane” hereafter) may exist. To date, the mechanisms underlying the biogenesis and physiological function of bulb structures remain unclear. To determine the biological significance of bulbs, we have addressed several lines of inquiry. We sought further structural evidence of bulbs; clues to its function; and direct evidence for a qualitative difference between the bulb membrane and the vacuolar membrane. Further structural evidence of bulbs has been provided from several previous studies that demonstrated the existence of bulb-like structures in a broad spectrum of tissue types and plant species.³⁻⁹ We also previously identified other markers associated with bulbs (Saito et al. 2011, Fig. 1C), based on the expression of own-promoter-driven GFP-VAM3, GFP-VTI11 and *Pro35S*-YFP-2xFYVE in bulbs. In addition, the cytoplasm of some bulbs was visualized with the expression of *Pro35S*-GFP. To elucidate the function of bulbs, we searched mutants with a bulb-less phenotype. We reasoned that a mutant with abnormally shaped vacuolar membranes may also be deficient in bulbs. Next, we found that some shoot gravitropism (*sgt*) mutants were nearly devoid

of bulbs in broad-type tissues, based on the expression patterns of own-promoter-driven GFP-VAM3, *Pro35S* YFP-2xFYVE and *Pro35S*GFP.¹⁰ We also provided direct evidence for a qualitative difference between the vacuolar membrane and the bulb membrane by visualizing the vacuolar membrane in a single cell with different colored markers, GFP-AtRab75 and TagRFP-Vam3. In this paper, we describe some unexpected results from this dual-color study of vacuolar membranes, the interesting conclusions that arose, and future perspectives.

Dual-color Visualization of Continuous Vacuolar Membranes with the Co-Expression of GFP-AtRab75 and TagRFP-Vam3

In our original report, we showed that γ -TIP-GFP expression could be used to visualize bulbs, but GFP-AtRab75 could not. However, in plants that expressed GFP-AtRab75, electron microscopy showed that the bulb structures existed.¹ To obtain direct evidence of qualitative differences between bulbs and peripheral vacuolar membrane, we introduced the *ProVAM3:TagRFP:VAM3* gene¹¹ into transgenic *Pro35S*-GFP-AtRab75 plants (GR7-10-1)¹ by cross-fertilization (Fig. 2A, B). The F1 progenies co-expressed TagRFP-Vam3 and GFP-AtRab75 (Fig. 2C). Unexpectedly, confocal laser scanning microscopy showed that, when co-expressed with TagRFP-Vam3, GFP-AtRab75 was also detected in bulbs (Fig. 2C). We then tested other conditions; e.g., the expression γ TIP-GFP and Venus-AtRab75 with a dual site gateway binary vector, or direct transformation of GR7-10-1¹ plants with a construct that harbored γ TIP-mRFP with other drug marker. In those conditions, the AtRab75 signal was also detected in the bulb

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Submitted: 09/08/11; Accepted: 09/13/11
DOI: 10.4161/psb.6.12.18061

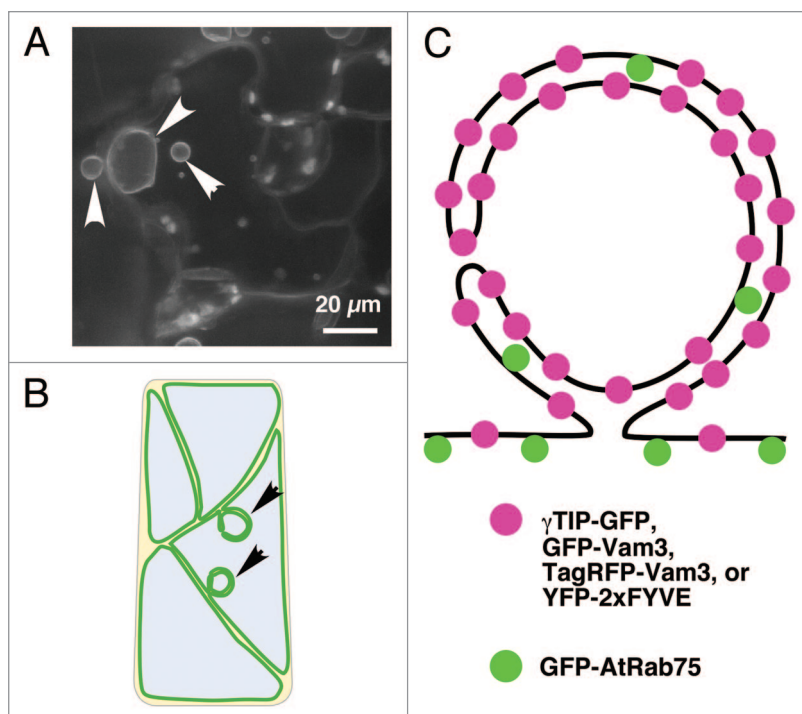


Figure 1. Structural evidence for bulbs based on the expression of other markers in plant cells. (A) Bulbs observed in Arabidopsis cotyledon. Confocal laser scanning microscopic images. White arrowheads indicate bulbs. (B) Schematic illustration of vacuolar membrane in plant cells. Black arrowheads indicate bulbs. (C) Model of a bulb.

(data not shown). We speculated that characteristics or contents of bulb membrane may change under the condition of the overexpression of membrane proteins (e.g., TagRFP-Vam3 or γ TIP-mRFP).

TagRFP-Vam3 is More Concentrated than GFP-AtRab75 in the Bulb

We noticed that the bulb membrane often appeared to be a deeper yellow than the peripheral vacuolar membrane when labeled with the dual color, TagRFP-AtVam3 and GFP-AtRab75 (Fig. 2D). This suggested that TagRFP-AtVam3 might be more concentrated in the bulb than GFP-AtRab75. To investigate this notion, we compared the fluorescence intensity/pixel of two markers that localized to the bulbs and to the peripheral vacuolar membrane (Fig. 2E). In the bulb, the average fluorescent intensity/pixel was 542 for TagRFP-AtVam3 and 1,078 for GFP-AtRab75 (Fig. 2E top). In the peripheral vacuolar membrane, the average fluorescent

intensity/pixel was 151 for TagRFP-AtVam3 and 633 for GFP-AtRab75 (Fig. 2E bottom). The ratio of the bulb: peripheral vacuolar membrane fluorescent intensity/pixel was $542/151 = 3.6$ for TagRFP-AtVam3 and $1,078/633 = 1.7$ for GFP-AtRab75. This suggested that TagRFP-AtVam3 was more concentrated than GFP-AtRab75 in the bulb. The bulb comprised two layers of vacuolar membranes. Thus, the fluorescent molecules that were distributed evenly over the both vacuolar membranes would emit a signal with approximately twice the intensity as the molecules that were only distributed over one of the membranes. Although the conditions in this study were artificial, this suggested that TagRFP-AtVam3 was concentrated in the bulb than GFP-AtRab75.

Conclusion and Perspectives

We propose that the complex configuration of vacuolar membranes may serve a specialized function. Our schematic model of the vacuolar subdomain is shown in Figure 3. In a recent report, we proposed that two independent mechanisms would be needed to maintain vacuolar integrity; this was based on the slightly different phenotypes of two bulb-less mutants, *ziglatvii11* and *sgr2*. The *ziglatvii11* mutant had no bulb structure and also had no transvacuolar strands; in contrast, *sgr2-1* had no bulb structure, but maintained transvacuolar strands. This suggested that the bulb biogenesis pathway might include two critical steps, one related, and the other unrelated, to the maintenance of transvacuolar strands. Furthermore, transvacuolar strands may form a distinct subregion within the vacuolar membrane (Fig. 3), because the membrane curvature of a transvacuolar strand is clearly different from the curvatures of other vacuolar membranes or transvacuolar strands; thus, we also speculate that bulbs have an unknown function that depends on the recruitment of specific molecules. Further studies of the molecular basis, function and biogenesis of bulbs will be necessary to understand what physiological roles bulbs play in the lifecycle of plants.

Disclosure of Potential Conflicts of Interest

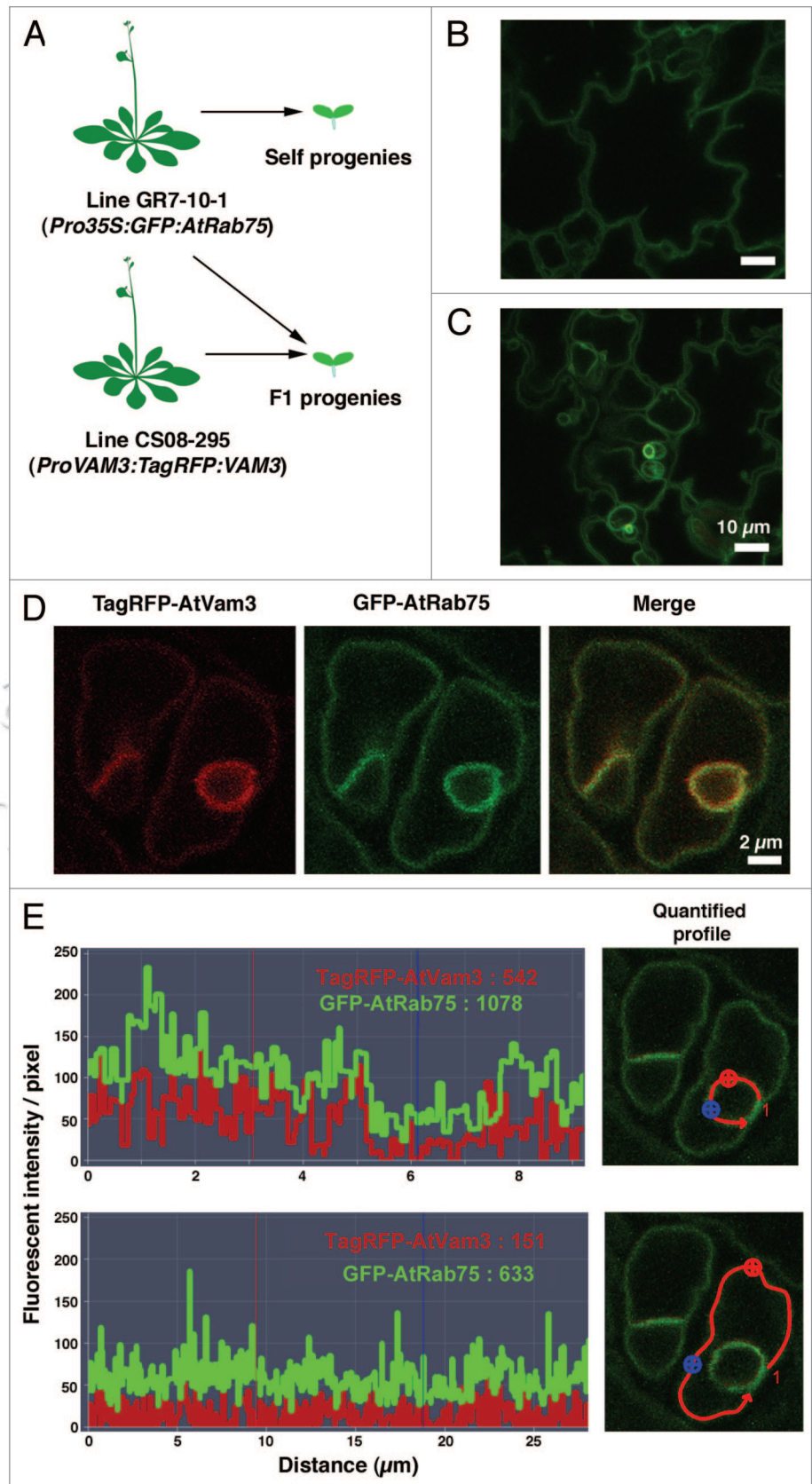
No potential conflicts of interest were disclosed.

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Figure 2. Dual color visualization of vacuolar membranes. (A) The generation of transgenic plants that express tagged proteins. (Top) Control self propagation is shown for the GR7-10-1 (Saito et al. 2002) plants. (Bottom) Cross-fertilization produced progeny that expressed both TagRFP:VAM3 and GFP:AtRab75. (B) Confocal laser scanning microscopic image of 3DAG cotyledon epidermal cells of plants produced by self progeny of GR7-10-1. (C) Confocal laser scanning microscopic image of 3DAG cotyledon epidermal cells of the F1 progeny of a cross between GR7-10-1 and *ProVAM3:TagRFP:VAM3* marker plant lines. (D) Confocal laser scanning microscopic images of guard cells of the F1 progeny of a cross between GR7-10-1 and *ProVAM3:TagRFP:VAM3* marker lines. (E) Quantification of fluorescent intensity/pixel in bulb membranes and peripheral vacuolar membranes. The areas quantified are indicated with red lines in the microscopic images on the right.

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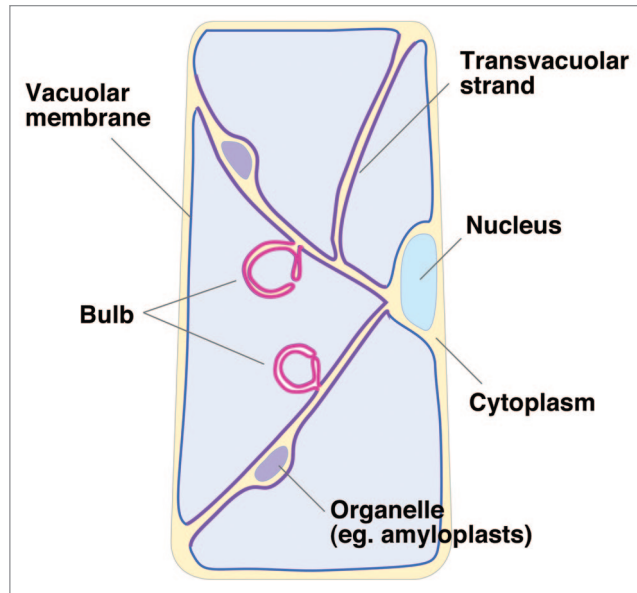


Figure 3. Schematic model of the vacuolar subdomain.

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