Tools to Study Plant Organelle Biogenesis. Point Mutation Lines with Disrupted Vacuoles and High-Speed Confocal Screening of Green Fluorescent Protein-Tagged Organelles[1][w]

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We have focused our studies in the past several years on understanding protein trafficking from the secretory system to the vacuole—an organelle present in all plant cells. Here, we report an approach for generating and screening plants with defects in vacuolar biogenesis. Plant vacuoles are commonly known to be multifunctional organelles, and recent findings have even demonstrated a variety of new roles for vacuoles and the vesicles that deliver cargo to them. Although it has always been assumed that vacuoles are essential for plant survival, the recent isolation of a T-DNA-tagged mutant called *vcl1* (*vacuoleless1*) has unequivocally demonstrated that vacuoles are vital organelles (Rojo et al., 2001). Mutations in the yeast (*Saccharomyces cerevisiae*) ortholog of *VCL1*, *VPS16*, also block vacuole biogenesis and affect all known vacuolar protein transport pathways in yeast; however, in contrast to VCL1, the *VPS16* gene product is not essential (Horazdovsky and Emr, 1993). A major effect of *VCL1* inactivation is that it blocks the formation of vacuoles, leading to embryonic lethality. Thus, although the isolation of *vcl1* served to emphasize the importance of plant vacuoles to plant growth and development, it is difficult to gain additional information about vacuole biogenesis from an embryo lethal mutant. Some important proteins that likely mediate trafficking to the vacuole are represented by single genes in the Arabidopsis genome. For example, each of the six members of the AtC-VPS complex for which VACUOLELESS1 is a member is encoded by a single gene (Rojo et al., 2003); thus, null mutations in these genes would also most likely be lethal.

Similar conclusions were drawn when several knockout mutants from the SNARE family were isolated. Although a T-DNA insertion into the *SYP61/OSM1* syntaxin is viable (Zhu et al., 2002), some reported null mutations of syntaxin genes are not tolerated by the plant. For example, a T-DNA disruption of a single member of the SYP2 and SYP4 gene families is gametophytic lethal (Sanderfoot et al., 2001). Another knockout mutant, "*knolle*" (*syp111*), is embryo/seedling lethal (Lukowitz et al., 1996). However, a point mutation in the *SYP22/SGR3* gene is viable, and the mutant lacks the shoot gravitropic response (Yano et al., 2003). Thus, it becomes apparent that the isolation of plants with point mutations would be a very valuable tool to isolate viable mutants for studying plant vacuolar biogenesis. With this in mind, we wanted to identify mutants with small defects in vacuolar biogenesis genes that at the same time would not be lethal to the plant.

To allow for effective visualization of vacuolar structure, we chose Arabidopsis lines expressing a fluorescent tonoplast marker, green fluorescent protein (GFP):δ-tonoplast intrinsic protein (TIP), under the control of the 35S promoter (Cutler et al., 2000). The tonoplast-localized GFP fusion protein in the tonoplast of these plants is easily visualized by confocal microscopy (Fig. 1, A–D). Homozygous seeds from 35S::GFP:δ-TIP plants were obtained, and vacuoles from these plants were isolated using the technique described previously by Ahmed et al. (2000). Proper GFP:δ-TIP localization at the tonoplast was confirmed by microscopy (Fig. 1E). Seeds from homozygous plants were then treated with ethyl methanesulfonate (EMS) to induce 1-bp changes throughout the genome of this line of plants. The Meridian Insight Point Confocal Microscope (Meridian, Okemos, MI) was used to screen 7-d-old seedlings from the M_2 generation for broken or malformed vacuoles, mistargeting of the GFP:δ-TIP chimeric protein, or other interesting phenotypes. The Meridian Insight Confocal has real-time ocular viewing confocal capability that allowed us to rapidly screen large numbers of seedlings. For each seedling, three types of tissues were examined: cotyledons, hypocotyls, and roots (Fig. 1A).

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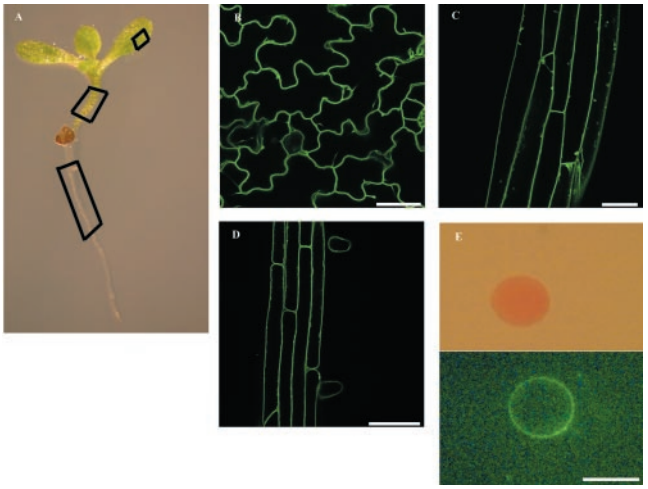

Figure 1. GFP:δ-TIP is expressed in the tonoplast of 35S::GFP:δ-TIP transgenic seedlings (A) such as cotyledon epidermal cells (B), hypocotyls (C), and roots (D). E, Example of an isolated vacuole stained with neutral red (top) and with GFP fluorescence (bottom). Scale bar = 40 μm.

Thus far, we have screened 9,175 M_2 EMS seedlings (56 pools; approximately 160 seedlings per pool) using confocal microscopy. Seedlings (620; 7%) showed mutations in pigment development, indicating that mutagenesis and the screening protocol were robust (Lightner and Caspar, 1998). Originally, 211 putative mutants with defects in vacuole biogenesis were obtained; however, 110 died before setting seed and/or did not produce any seed (Fig. 2, I–L), resulting in our current population of 101 putative vacuolar mutants. These mutants have been sorted into four broad categories based on their subcellular phenotypes in the M_2 generation.

The first category of mutants (*bub* [bubble-bath]) is characterized by increased numbers of small vacuolar vesicles in the cell (Fig. 2, A–C). Forty-six plants fell into this category. Among these plants, the *bub* phenotype was observed either in roots (three plants), in cotyledons (29 plants), or in hypocotyls and cotyledons (six plants). Although *bub* plants appear to have a large, central vacuole, they also have increased numbers of vesicles decorated with GFP:δ-TIP relative to the parental line. The severity of the phenotype correlated with plant lethality because plants with the highly pronounced *bub* phenotype did not survive (Fig. 2, I–K) or did not produce seeds. The second category of mutants contained large aggregates of GFP fluorescence (*agg*; Fig. 2, D–F). Thirty-four plants fit into this category. Among these plants, the *agg* phenotype was observed either in roots (14 plants), in hypocotyls (nine plants), in cotyledons (one plant), in hypocotyl and cotyledons (one plant), or in hypocotyls and roots (one plant). Upon closer examination of M_3 *agg* plants, we observed that some of the aggregates are membrane-bound compartments containing clusters of vesicles (Fig. 2F). The third category contained mutants that showed vacuoles apparently transected by transvacuolar strands (*tvs*; eight plants; Fig. 2, G and L). Among these plants, the *tvs* phenotype was observed either in roots (three plants), in cotyledons (two plants), in hypocotyl (one plant), in hypocotyl and cotyledons (one plant), or in hypocotyls and roots (one plant). Further investigation of the M_3 generation of *tvs* plants revealed that their vacuoles were extremely dynamic with the continuous rearrangement of the transvacuolar strands. Additional mutants appear to have unique and more complex phenotypes (Fig. 2H). Thirteen viable M_2 plants were clustered into this category. The unique phenotypes included defects in the regular pattern of the cotyledon epidermal cells (seven plants). Interestingly, approximately one-half of seedling-lethal mutants (60

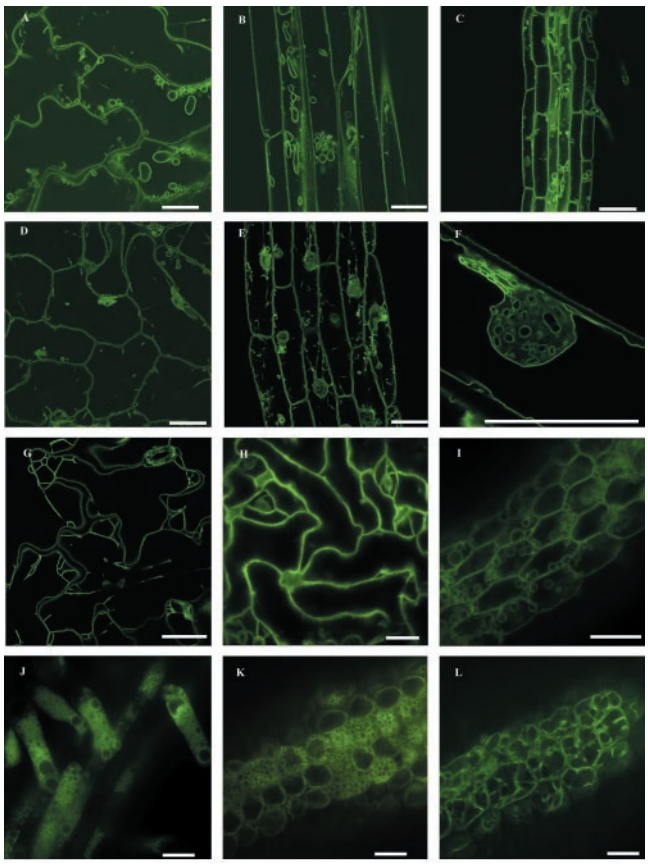

Figure 2. Examples of vacuolar mutants identified. *bub* mutants have increased numbers of vesicles in the cotyledons (A), hypocotyls (B), or roots (C). Examples of *agg* mutants with aggregates in the cotyledons (D) and hypocotyls (E). A closer look at the aggregates (F) reveals a membrane-bound vesicular structure. G, Example of the *tvs* mutant class with increased transvacuolar strands. Some of the mutants had complex phenotypes, such as disruption of cell shape (H). I to L, Many interesting M_2 vacuolar mutants that did not survive to the M_3 generation. Images A to G were collected by a Leica TCS SP2/UV Confocal Microscope (Leica Microsystems, Wetzlar, Germany). Images H to L were collected on a Meridian Insight Point Confocal Microscope with a CCD-cooled camera. Scale bar = 40 μm.

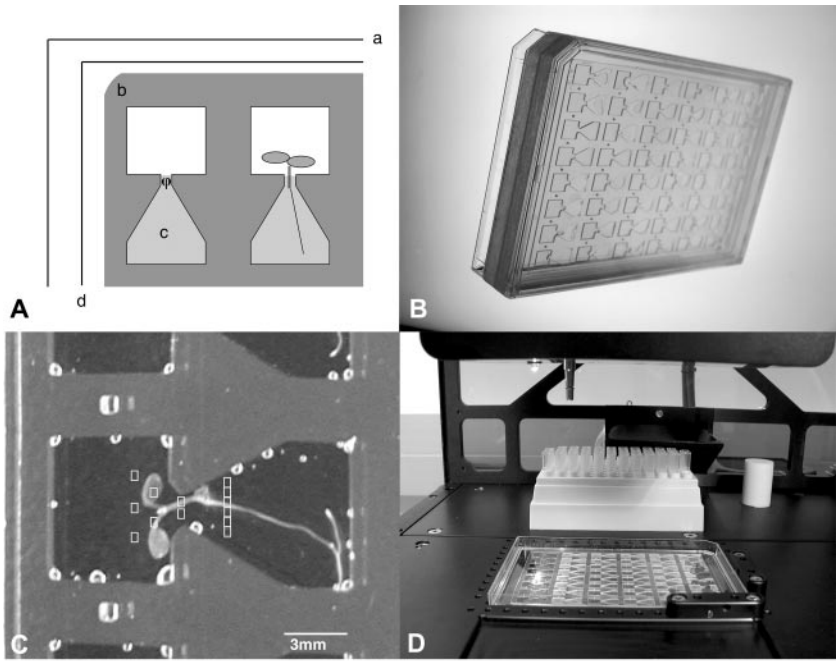

Figure 3. A, Assembly of Screening Plates: a standard multiwell plate lid (a) has a silicone gasket with 48 hourglass-shaped holes (b) applied to its outer surface. The bottom half of each opening (c) is filled with solid growth medium, and a seed is pipetted into the neck. A sheet of cellophane (d) flattens and seals the screening wells while allowing gas exchange. The cellophane is replaced with a coverslip for imaging. Populated plates are stacked together, sealed with surgical tape, and then incubated in a vertical position for 7 d. To keep the seeds hydrated, a layer of agar is deposited on the inside of each plate before it is populated and stacked. B, Populated plates viewed from above. Gravitropism assures vertical orientation of seedlings, which are illuminated evenly from all sides. C, Seedling in single well. The squares indicate the standard 5 + 2 + 6 search pattern for automatically finding cotyledon, hypocotyl, and root, respectively. Scale bar = 3 mm. D, Plate in the climate controlled imaging chamber of the Pathway HT automated imager. All tissues are close enough to the cover glass to be imaged by the UApo/340UV 20×/0.75NA objective lens, which moves on linear motors below the sample. For more information on microscopy methods described above, visit http://www.cepceb.ucr.edu.

plants; Fig. 2, I–L) showed complex or unique phenotypes throughout the seedling.

Because genetic mapping of these mutants will involve large numbers of F_2 seedlings, we are working toward developing a high-throughput screening process. We are now using an Atto Pathway HT high-throughput confocal microscope system (Atto Bioscience, Rockville, MD) with culture plates of our own design for germinating and growing seedlings (Fig. 3). All tissues are screened manually or by an advanced automated imaging routine without damaging the seedlings (Fig. 3, C and D). An example of the wild-type and mutant (*bub*) images produced by the Atto Pathway microscope can be accessed in supplemental data, available in the online version of this article at http://www.plantphysiol.org.

CONCLUSIONS

Using a mutagenized transgenic line expressing a tonoplast-localized protein fused to GFP, we were able to screen for vacuolar biogenesis mutants using confocal microscopy. Although we used EMS to mutagenize plants to generate point mutations, approximately 50% of the vacuolar mutants did not survive. Nevertheless, we were able to isolate four groups of mutants that would be useful in further analysis of vacuolar biogenesis. In our mutant screen, we found mutants that exhibit defective or modified vacuoles throughout the plant and mutants whose vacuolar phenotype is specific to a particular tissue of the plant. This suggests that the endomembrane system in shoots can be uncoupled from organization of the endomembrane system in roots and indicates that vacuolar biogenesis has tissue-specific components. It is also important to note that we never recovered a mutant seedling that completely lacked a large, central vacuole (Fig. 2, A–L). This result supports previous conclusions by Rojo et al. (2001) that the vacuole is an essential organelle to the plant cell. It is likely that an approach encompassing only transient disruptions of vacuolar biogenesis components, such as chemical genetics, will be beneficial to directly target many fundamental vacuolar biogenesis proteins.

Although the screen was originally performed using the Meridian Insight Point Confocal Microscope, a system has been developed that grows up to 48 seedlings on a multiwell plate lid, which can be imaged automatically. Although fully automating the process is at an early stage of development, it will

eventually increase the scope of possible experiments, including chemical genetics screens to test for the effects of drugs on seedling germination and tissue development. One of the challenges will be to manage the large volume of data generated by automated screening. Similar screening approaches could offer an excellent opportunity to study the biogenesis of other plant organelles.

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