Multiple Vacuoles in Plant Cells

After one retires, it is a pleasant surprise to learn that one’s prior publications are being mentioned in the current literature. In this regard, two articles in the December 2007 issue of *Plant Physiology* pay me the compliment of challenging some of our published work, and it is my honor to respond. Hunter et al. (2007) and Olbrich et al. (2007) separately challenge the hypothesis that plant cells contain functionally distinct types of vacuoles, in which tonoplast intrinsic protein (TIP) isoforms serve as markers for specific functional types. It is my view that the challenges are unsuccessful and that the plant cell biology community would benefit from a brief discussion of the problems with those articles.

Hunter et al. (2007) argue that results in Arabidopsis (*Arabidopsis thaliana*) contradict the multiple vacuole hypothesis. They present results indicating that, by expression of fusions of yellow fluorescent protein (YFP) with α-, γ-, and δ-TIPs in transgenic Arabidopsis, driven either by the cauliflower mosaic virus 35S promoter or the respective endogenous TIP promoter, they cannot identify separate vacuolar compartments. Thus “TIP isoform distribution is tissue and development specific, rather than organelle specific” (p. 1371). They further claim, based on their fusion protein results and on publicly available microarray data, that “only α-TIP is present during embryo maturation and germination. This is in apparent contrast with results obtained by immunofluorescence in Arabidopsis seeds, using peptide antibodies against the δ-TIP” (p. 1379). Results from western blots of extracts from mature seeds and germinating seeds probed with anti-GFP antiserum were stated to support this claim. Thus, the authors appear to argue that Arabidopsis is different from other plants in which traffic to vacuoles has been studied. However, there is strong literature support for the concept that sorting of proteins to vacuoles in Arabidopsis is not different from that described in other species. Using quantitative immuno-electron microscopy (EM) analyses of the distributions of storage proteins, the Cys protease aleurain, the BP80/VSR (vacuolar sorting receptor), and the RMR protein (Park et al., 2007), Hinz et al. (2007) demonstrated in developing Arabidopsis embryos that storage proteins partitioned into dense vesicles with the RMR protein, whereas the BP80/VSR protein was largely associated with clathrin-coated vesicles. These results, and the finding that storage protein sorting initiated by aggregation early in the Golgi, were fully consistent with previous studies of developing pea (*Pisum sativum*) embryos (Hinz et al., 1999; Hillmer et al., 2001), and with data strongly supporting the concept that RMR proteins are sorting receptors for proteins carrying C-terminal vacuolar sorting signals (Park et al., 2005, 2007). The claim by Hunter et al. (2007) that it was not possible to visualize organelles in vegetative Arabidopsis tissues, in which separate sorting of soluble proteins for the lytic and storage protein pathways could be identified, is at odds with results presented in another article in the same December 2007 edition (Poustka et al., 2007).

Additionally, the interpretation of Hunter et al. (2007) is at odds with published data demonstrating a key role for a conserved sequence motif in the cytoplasmic C terminus of α-TIP in targeting it to its unique pathway to a vacuole destination (Oufattole et al., 2005). In those experiments, a fusion of GFP with the N terminus of α-TIP was transiently expressed in tobacco (*Nicotiana tabacum*) suspension culture protoplasts. The wild-type protein clearly localized to small vacuoles that were separate from the central vacuole, whereas a mutant protein lacking the conserved cytoplasmic sequence underwent altered proteolytic cleavage and was additionally present on the central vacuole tonoplast (Oufattole et al., 2005). These considerations are relevant because the transgenic Arabidopsis plants studied by Hunter et al. (2007) expressed C-terminal fusions of YFP to the TIPs. Although the authors studied the localization of both the N-terminal and C-terminal fusions, the possibility cannot be eliminated that C-terminal fusions may have altered the targeting. Overexpression of our α-TIP fusion proteins appeared to be toxic to the protoplasts (Oufattole et al., 2005), a fact that would not be surprising considering that it presumably actively functioned as an aquaporin and might well have altered vacuolar volume or composition adversely. Although a portion of the results of Hunter et al. (2007) used expression from the native TIP promoters, in effect this would still result in overexpression of the respective proteins because the transgene expression would be added on top of the endogenous gene expression. Their western-blot results strongly indicate that this probably was detrimental to the plants (supplemental figure S5B from Hunter et al. [2007]). There, the anti-GFP antibodies detect only a protein the expected size of GFP itself, not of a GFP-TIP fusion protein. This would be explained if the altered vacuole membrane containing an excess of TIP protein were targeted for degradation. Indeed, internalization of vacuole membrane is associated with the protein aggregation/membrane internalization pathway followed by RMR proteins (Jiang et al., 2000; Oufattole et al., 2005); when a fusion protein in which the RMR cytoplasmic tail tagged with GFP was expressed, the cytoplasmic portion was internalized such that it was proteolytically cleaved to release intact GFP that was visualized in the central vacuole lumen (Park et al., 2007).
Finally, the statement that γ- and δ-TIPs are not present in maturing seeds was not tested in any of their experiments and is therefore unjustified. The microarray data indicate that expression of those mRNAs is high early in embryo development but then declines as the seed matures. Those data do not address the abundance of the proteins. Poxleitner et al. (2006) presented detailed western-blot analyses of extracts from mature dry and germinating Arabidopsis seeds; those data clearly show the presence of α- and δ-TIPs in mature seed extracts, with a weaker but detectable signal for γ-TIP. When localized in sections of mature Arabidopsis embryos by immunofluorescence, γ-TIP was associated with internal structures in protein storage vacuoles (PSVs; Gillespie et al., 2005). This is consistent with our hypothesis that PSVs are a type of multivesicular body where separate compartments contain lytic and storage components, and where γ-TIP is a marker for the internal, lytic compartment (Jiang et al., 2001, 2002).

Olibrich et al. (2007) essentially argue that they were unable to reproduce our findings of different vacuole types within barley (Hordeum vulgare) and pea root tips (Paris et al., 1996; Jauh et al., 1999; Moriyasu et al., 2003). Their first approach was to embed fixed root tips in wax, cut sections, label with a single antibody, and then view by epifluorescence. Based on the patterns of labeling for each antibody, they conclude that “root tips of young barley and pea seedlings do not develop separate populations of PSV- and LV-[lytic vacuole]-type vacuoles” (p. 1390). Immunofluorescence provides the ability to screen large numbers of cells through their full volume in a short time, thereby allowing one to focus on those where distinct structures are present. However, it is puzzling that double-label studies were not done—how else can one evaluate the possibility of different populations of organelles within cells? Additionally, as quantified in previous work (Jauh et al., 1999), much of the root tip cell population had large vacuoles in which multiple TIPs were present—clear distinctions were observed primarily in the cells with small vacuoles, and those were a minor part of the population. That was the basis for our hypothesis that vacuole biogenesis was developmentally regulated, where a large central vacuole with multiple TIP markers was an end product. Visualization of structures labeling for individual TIP isoforms was optimized by using confocal microscopy, a technique not used here. If one wants to argue that previously published results are incorrect, one should repeat the experimental protocol used in those studies. Use of double-label EM is not comparable. Neglecting the consideration that glutaraldehyde fixation might alter the protein epitopes available to the antipeptide antibodies, by EM one can study a very few structures in a very few cells. Additionally, the labeling protocol was not set up to allow quantitative comparison of protein abundance in different vacuoles—it is a reasonable guess that the authors would not have been able to measure even a 10-fold difference in abundance of the different TIP isoforms. The level of discrimination in their studies was whether any labeling was present.

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