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Chapter 11

Volume measurement of guard cell vacuoles during stomatal movements using confocal microscopy

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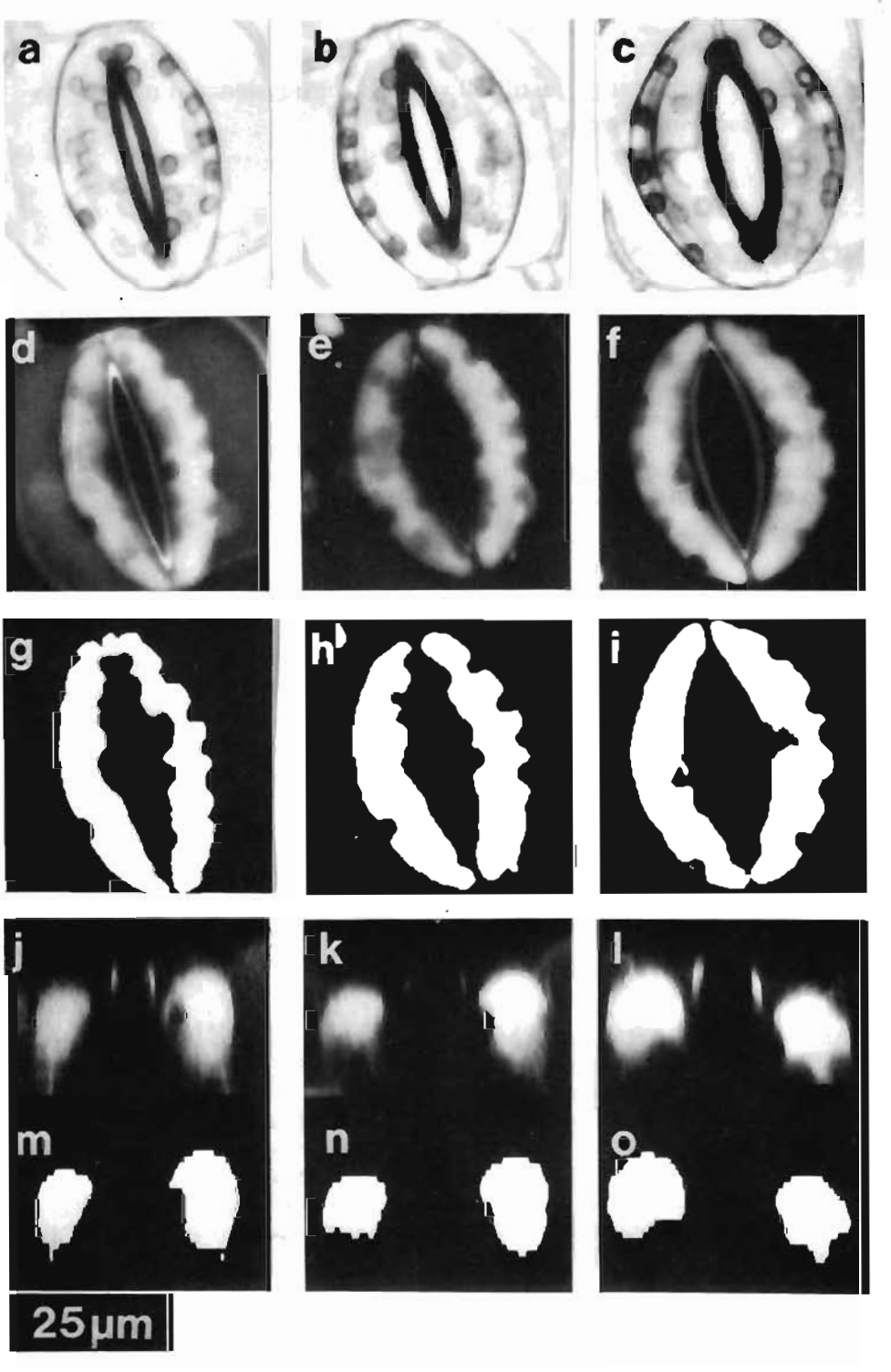
Abstract. The volume of the guard cell vacuole was measured during stomatal movements in *Commelina* after vital staining with the fluorochrome acridine orange. Serial optical sections were taken at time intervals during stomatal opening or closure with a confocal scanning laser microscope. Volumes were calculated from summation of the vacuole areas detected by image analysis techniques in each section.

1. Introduction

Stomata are small pores in the leaf epidermis surrounded by two guard cells. Changes in guard cell shape, and hence pore area, control gaseous diffusion in and out of the leaf and serve to balance CO_2 uptake for photosynthesis against H_2O loss via transpiration. Stomatal movements result from a reversible, turgor-driven deformation of the highly specialised guard cells. Description of the geometry of the stomatal complex is laborious using conventional fixation and sectioning techniques, which, in addition, cannot resolve dynamic changes within and between living cells ocurring during stomatal movements. Determination of the volume of cellular compartments also enables accurate prediction of changes in ion concentrations and fluxes from tracer flux analysis or ion selective micro-electrode studies. The optical sectioning capability of the confocal scanning laser microscope (CSLM) was used in this study as a rapid means to determine directly the morphology and volume of the guard cell vacuole during stomatal movements.

2. Materials and Methods

Abaxial epidermal peels from *Commelina communis* were prepared according to Fricker and Willmer (1987) and maintained in a continuous perfusion system as described by Gilroy et al., 1990. The incubation medium (50 mol m^{-3} KCl, 1 mol m^{-3} MES, pH 6.1 with KOH) was bubbled with CO_2-free air at 19°C. Flow rates were 80 mm^3 s^{-1}. Illumination (420 ± 20 nm, 90 µmol m^{-2}) was provided by suitable filtration of the 'microscope transmission light source. Vacuoles were stained with the vital dye acridine orange (AO, 1 mmol m^{-3}, 10 min). Apertures were manipulated by varying the concentration of KCl between 25 and 100 mol m^{-3}. Optical sections were obtained using a BioRad MRC 500 CSLM in fluorescence mode (488 nm excitation) coupled to a Nikon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Diaphot microscope via a modified side port adaptor. Specimens were observed using a x 60, 1.4 N.A., oil immersion objective. Guard cell vacuoles accumulate the dye and fluoresced more strongly than vacuoles of other cells in the epidermis. There was negligible chloroplast autofluorescence, however, signals from the pore lip and nucleii required masking in some sections. The second channel of the MRC 500 was used as a transmission detector coupled to the microscope condenser by a fibre-optic cable. 3-D data sets were recorded stepping xy sections through z plane at 1 μm increments. Images were averaged over 16 frames and median filtered (5 x 5). The same cell was sampled at 30 min intervals. Complete 3-D series were output for processing using the Semper6+ language. Stomatal pore widths were measured from a projection of the transmission images using a linear sum of frames algorithm, effectively giving a bright field image.

3. Results and Discussion

The morphological changes associated with stomatal opening are shown in Figure 1 a-c as projected xy transmission images. The summed xy projections of the confocal AO fluorescence image series taken in parallel, are shown in Figure 1 d-f, along with a single median xz section (Figure 1 j-l). The vacuole appeared as a single compartment, negatively staining other organelles, particularly the nucleus and chloroplasts. More than one vacuole was rarely observed, though during rapid closure the vacuole occasionally fragmented and sometimes appeared to pinch off small vesicles into the lumen.

The vacuolar volume was determined in three stages:
(1) Selection of a threshold defining the edge of the vacuole.
(2) Manual removal of extraneous signal from the detected image.
(3) Summation of the detected pixels assuming each represented the volume of a rectangular voxel extending half the section thickness above and below the image plane.

The threshold was determined as the region of minimum overlap between the frequency distribution of pixel intensities attributed to fluorescence from the guard cell vacuole versus other signals (Figure 2).

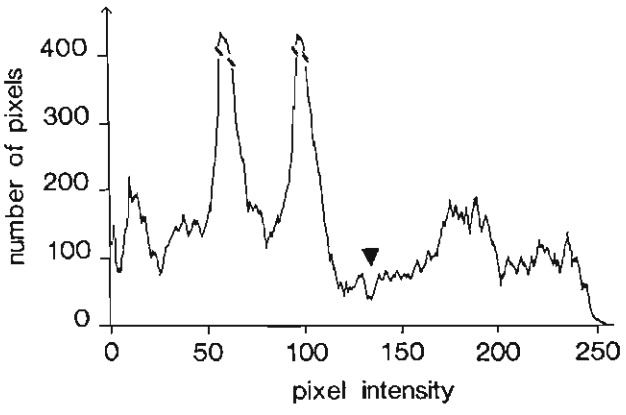


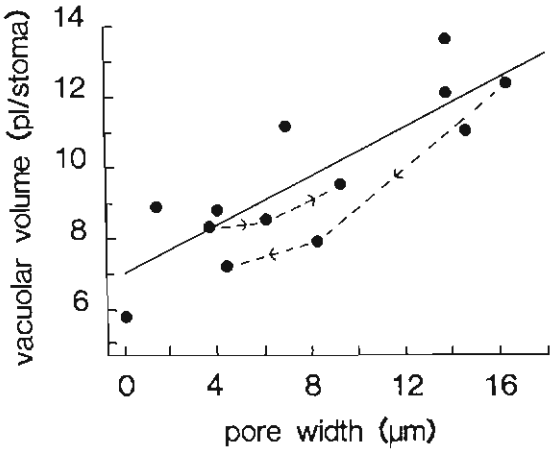
Figure 2: Frequency histogram of pixel intensities in the fluorescent image from the median xy plane. The point of minimum overlap was used as a threshold to detect vacuolar regions.

Typical results after thresholding the mid xy section using this value are shown in Figure 1, g-i and the mid orthogonal section through all the thresholded images in Figure 1, m-o.

No correction for blurring associated with the point spread function or scattering and absorption within the specimen was applied prior to thresholding, however, to give some indication of the sensitivity of the final volume measured to error introduced at this stage, volumes were also calculated with an imposed ± 10% deviation from the set threshold value (Table I).

Table I

	Vacuolar volume (pl)		
pore width µm	3.7	6.1	9.2
threshold:			
-10%	7.72	8.01	8.80
as set	8.30	8.51	9.50
+10%	8.85	9.16	10.1



The measured vacuolar volume increased by 14.4 % during opening over 5.5 µm. The volume measurements were relatively insensitive to imposed 'error' in the value of the threshold used, varying less than 7% with a 10% change in threshold in all cases. The relationship between vacuolar volume and pore width was determined by linear regression (Figure 3). Dashed lines link measurements made on the same stoma, arrows indicating the direction of movement. Volume = 6.99 + 0.345 x aperture; corr. coeff. = 0.84; R^2 = 70%.

No other direct measurements of guard cell volumes have been previously made for comparison with this data, however, the slope of the regression equation derived here closely parallels estimates for the change in *total* guard cell protoplast volume during stomatal movements measured from Nomarski images (e.g. Raschke, 1975; MacRobbie and Lettau, 1980). Interestingly the estimated cytoplasmic volume per guard cell (total - vacuolar) using data from MacRobbie and Lettau (1980) increased from 0.6 to 0.9 pl over the range 5 - 15 µm. We are currently extending these studies to measure total protoplast, wall and organelle volumes using this technique.

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