Real time studies of cell-to-cell communication

P. Boevink, J. Calcutt, A.G. Roberts & K.J. Oparka

Effective co-ordination of the growth and behaviour of plant cells is essential for normal development. The main effectors of this co-ordination are a wide variety of signalling molecules, such as ions, sugars, RNAs, proteins and metabolites. A major pathway for the transmission of such signalling molecules from cell to cell is through plasmodesmata, complex, membrane-lined channels that connect most plant cells with their neighbours. Signal transmission through plasmodesmata is potentially very rapid and direct as both the endoplasmic reticulum (ER) and cytoplasm are continuous between cells through their plasmodesmata. Plasmodesmata, however, are not open pipes through which molecules flow freely. Instead, they are filled with proteins and cytoskeletal structures, and their openings are often constricted. Small ions and sugars are thought to diffuse through plasmodesmata, but how do the larger molecules such as RNAs and proteins pass through?

Both electron microscopy tissue preparation and microinjection techniques are likely to affect plasmodesmata, as they are very sensitive to changes in the cell, so we cannot be sure that the size exclusion limit (SEL) values determined reflect the natural state. Furthermore, we have very little information on the dynamic properties of plasmodesmata. We do not know whether macromolecular trafficking through plasmodesmata involves a general opening of the channel (gating) or might, in addition, require chaperone proteins. The use of fluorescent proteins has led to many advances in our understanding of plasmodesmal function. We have shown by bombardment of plasmids expressing variously sized fluorescent protein fusions that the SEL of plasmodesmata in sink regions of the plant are much greater than in source regions[1]. These data were not real-time, however, as it takes at least 6 hours for sufficient proteins to be produced in the bombarded cell for it to be detectable. Recently, a new tool has been developed that has overcome this limitation. Photoactivatable GFP (PA-GFP) was developed by Patterson and Lippincott-Schwartz[2]. It is a variant of a bright GFP (EGFP) that is barely fluorescent until activated by a burst of approximately 400 nm wavelength light, after which it becomes highly fluorescent.

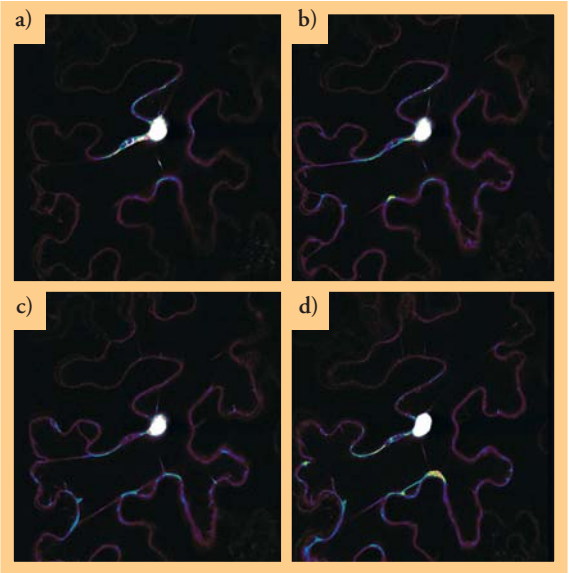

Figure 1 Photoactivation and spread of PA-GFP within a cell. An area within the nucleus of this large, mature *Nicotiana* epidermal cell was photoactivated and the movement of the activated PA-GFP from the nucleus to the cytoplasm was imaged for 20 scans (about 2 minutes). These four images were taken from that series; (a) was the first image and (d) was the last, (b) and (c) are from within the series, the four images are evenly spread over time. They are false coloured such that the most intense fluorescence appears white while low intensity fluorescence appears purple. The images are single optical sections. All images in this article were taken with a Leica SP2 confocal microscope.

We have expressed PA-GFP transiently in *Nicotiana* species by agroinfiltration, and constitutively in transgenic *Arabidopsis* produced by the floral dipping method. The PA-GFP was activated in the nuclei of expressing cells by single scans of regions of interest (ROIs) at maximal zoom (x32) with 405 nm light from a blue diode laser. Nuclei were chosen because they contain high concentrations of fluorescent protein, allowing more accurate activation of individual cells. Activated PA-GFP moved out of the nucleus into the cytoplasm almost instantly and then became rapidly distributed throughout the cell (Fig. 1). Extensive movement of PA-GFP from activated cells on the smallest agroinfiltratable leaves was observed, but it was restricted to the activated cell in the majority of mature source leaves. Interestingly some mature cells did allow movement of PA-GFP into one or two neighbouring cells (Fig. 2a). This suggests that at any one time the plasmodesmata linking mature cells may be gated (have increased SELs) to allow free exchange of information. Movement was most commonly observed into and out of subsidiary cells adjacent to stomata.

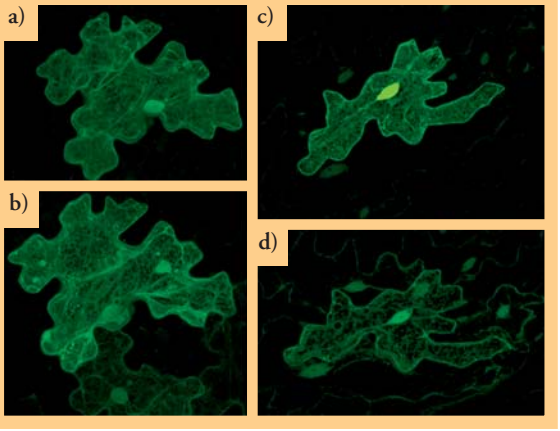

Figure 2 Movement of PA-GFP in mature *Nicotiana* and *Arabidopsis* leaves. An epidermal cell on a *Nicotiana tabacum* large source leaf a couple of minutes after activation (a) and 21 hours later (b). Some movement of the fluorophore has occurred into two cells abutting the activated cell (in the lower half of the image in b) but not into all adjacent cells. In contrast the PA-GFP activated in this apparently mature *Arabidopsis* epidermal cell (c) has moved into all surrounding cells in 6 hours (d). Images are composed of serial optical sections.

The results of activation of PA-GFP in the transgenic *Arabidopsis* leaves suggest that the sink-source transition pattern is very different from that in *Nicotiana* species. *Arabidopsis* leaf cells appear more 'leaky', that is the activated PA-GFP moves extensively within an hour, even on apparently mature leaves (Fig. 2b). Unlike constitutively fluorescent proteins, PA-GFP allows the movement of the protein from activated cells to be quantified. Measurement of the PA-GFP fluorescence in symplastically isolated guard cells confirmed that there was no movement on PA-GFP when plasmodesmatal connections were absent, and revealed a slow degradation of the PA-GFP of around 16% in 18 hours. This latter result suggests another use for PA-GFP, namely in the analysis of protein degradation within plant cells. A protein of interest could be fused to PA-GFP, activated, and the kinetics of degradation determined in real time by measuring the loss of fluorescence.

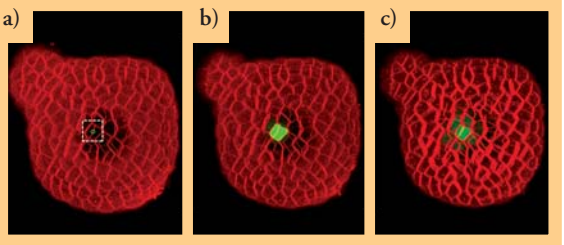

Figure 3 Photoactivation in *Arabidopsis* shoot meristems. A single cell at the top of a shoot apical meristem was phoactivated by scanning the area within the green circle shown in (a) at maximum zoom, the broken white line indicates the maximum zoom. An image was taken immediately after activation (b) and again after 2 hours (c). The fluorescence from the activated cell has diffused into surrounding cells. Images are composed of serial optical sections.

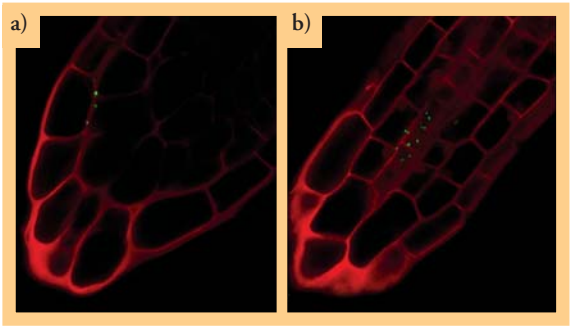

Figure 4 30K-PAGFP activation in roots. Activation of 30K-PAGFP in plasmodesmata of individual side (a) and facing (b) walls in two *Arabidopsis* roots. Images are single optical sections.

Signal exchange between cells in meristems is essential for normal development of plant tissues. Transcription factors that regulate development, such as Knotted-1, are thought to move between specific cell layers in the meristem via plasmodesmata. PA-GFP transgenic *Arabidopsis* will provide us with the first opportunity to study macromolecular trafficking in meristems non-invasively and in real time. In preliminary work PA-GFP has been activated in specific cells in root and shoot meristems (Fig. 3).

A crucial factor in the ability of molecules to move through plasmodesmata lies in the structure of the plasmodesmal pore. Simple plasmodesmata, which are predominantly found in immature tissues, have significantly greater SELs than branched plasmodesmata. The 30K movement protein from TMV is a useful tool for monitoring the development of plasmodesmata. The 30K protein only accumulates (as a fluorescent protein fusion) in branched plasmodesmata. We have generated transgenic *Arabidopsis* expressing the 30K protein fused to PA-GFP (Fig. 4). Activation of fluorescence in specific plasmodesmata in dividing cells will allow us to monitor changes in the locations of plasmodesmata during and after cell division.

PA-GFP, the first robust activatable fluorescent protein has great potential for non-invasive studies of intercellular communication in plants and will also be a powerful tool for studies of intracellular protein dynamics.

References

[1]Oparka, K.J., Roberts, A.G., Boevink, P., Santa Cruz, S., Roberts, I.M., Pradel, K.S., Imlau, A., Kotlizky, G., Sauer, N., & Epel, B. (1999) *Cell* **97**, 743-754

[2]Patterson, G.H. & Lippincott-Schwartz, J. (2002) *Science* **297**, 1873-1877