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## Model System for Plant Cell Biology: GFP Imaging in Living Onion Epidermal Cells

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### ABSTRACT

The ability to visualize organelle localization and dynamics is very useful in studying cellular physiological events. Until recently, this has been accomplished using a variety of staining methods. However, staining can give inaccurate information due to nonspecific staining, diffusion of the stain or through toxic effects. The ability to target green fluorescent protein (GFP) to various organelles allows for specific labeling of organelles *in vivo*. The disadvantages of

*GFP thus far have been the time and money involved in developing stable transformants or maintaining cell cultures for transient expression. In this paper, we present a rapid transient expression system using onion epidermal peels. We have localized GFP to various cellular compartments (including the cell wall) to illustrate the utility of this method and to visualize dynamics of these compartments. The onion epidermis has large, living, transparent cells in a monolayer, making them ideal for visualizing GFP. This method is easy and inexpensive, and it allows for testing of new GFP fusion proteins in a living tissue to determine deleterious effects and the ability to express before stable transformants are attempted.*

### INTRODUCTION

The visualization of organelles, proteins and cellular dynamics is useful for understanding cellular physiological

events, including cell signaling. Organelles and proteins are traditionally imaged using various staining methods and different modes of fluorescence microscopy. However, staining methods can provide inconclusive results due to nonspecific staining, diffusion of the stain and suboptimal resolution. Some stains can prevent the observation of organelle dynamics through cross-linking or toxic effects. With recent improvement of the green fluorescent protein (GFP) for use in living plants (10), transient and stable gene expression methods are being exploited to study various plant cell organelles (13).

Transient gene expression is typically achieved following electroporation or bombardment into suspension culture cells (2) or protoplasts (31). However, suspension and protoplast cultures require hormones and are time-consuming and expensive to produce. Suspension culture cells such as tobacco

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BY2 cells require auxin and/or cytokinin in the culture media. The presence or absence of particular compounds in the media might influence organelle structure and dynamics. An example of this is the ability to synchronize Golgi differentiation in tobacco BY2 cells by auxin deprivation (31). Protoplasts lack the “support” normally provided by the cell wall and the cytoskeletal-extracellular matrix connections, which renders them very fragile and might cause organelle patterns different from those seen in whole cells.

Stable transformation of GFP fusions is widely used and has been instrumental in visualizing and studying the endoplasmic reticulum (ER) and Golgi (5,24), the plant mitochondria (14) and the nucleus (9). Transgenic plants expressing GFP allow analysis of cellular structure, but present several other problems, including the following: (i) Stable transformation is a time-consuming, expensive process; (ii) The plants typically used for stable transformation (i.e., *Nicotiana* and *Arabidopsis*) have small cells that render visualization of intracellular compartments difficult; (iii) Using a whole plant for visualization of a fluorescent protein presents out-of-focus information from the surrounding cells unless sectioning and/or confocal microscopy is used. In addition, structures within whole plants (e.g., vascular tissue and chloroplasts) can be autofluorescent.

Cells from epidermal peels of onion (*Allium cepa*) have been used as a model system for light microscopy. Because they are large, relatively transparent and occur naturally as a monolayer, these cells are particularly advantageous. As a model system, onion cells have proven instrumental in the study of cellular structures, including the plasma membrane (20) the ER (1,17,27), the nucleus (16), callose deposits (4), the cytoskeleton (2,15,26,32), plasmodesmata (20), and Hechtian threads (23). Onion cells have also been useful in studying cell processes such as cytoplasmic streaming (28), endocytosis (21), secretion (19), mitosis (29), plasmolysis (18), effects of osmotic stress on the plasma membrane (18) and characterization of mechano-sensory channels (7). In this paper, we describe their use as a rapid, transient gene expres-

sion system, ideal for subcellular localization of organelles and organellar dynamics in whole living cells. In particular, we used onion epidermal cells as a transient gene expression system for imaging GFP localized to different cellular compartments. Particle bombardment of onions as a transient expression system is equally applicable to the localization of previously uncharacterized proteins.

## MATERIALS AND METHODS

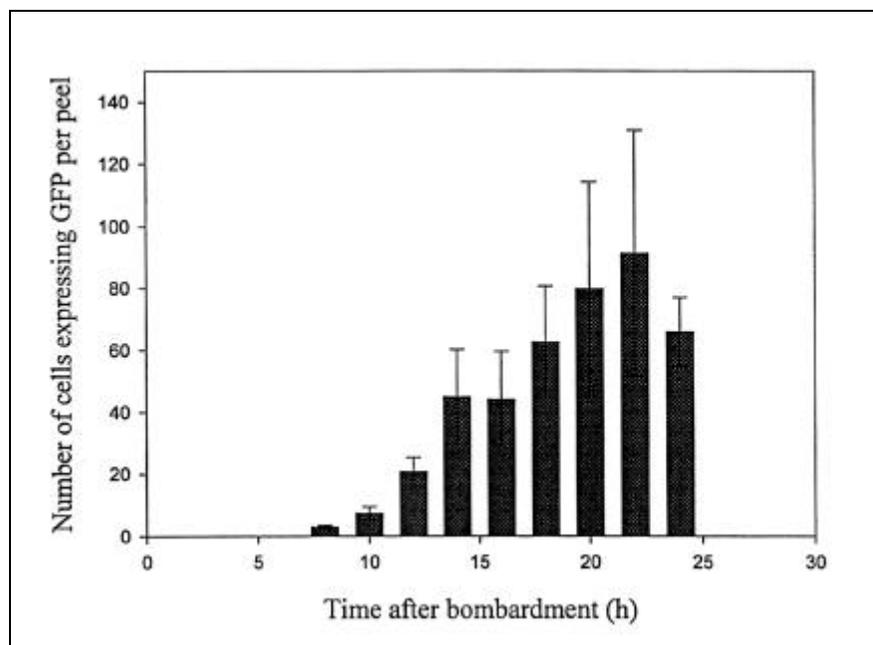
### Plant Material

*Allium cepa* cv. Tango bulbs were purchased from Martin Produce (Greely, CO, USA). Under sterile conditions, inner epidermal peels (2 × 2 cm) were placed on agar plates containing 1 × Murashige and Skoog (MS) salts, 30 g/L sucrose and 2% agar (Type IV; Sigma, St. Louis, MO, USA), pH 5.7. Peels were bombarded within 1 h of transfer to agar plates.

### GFP Constructs

GFP constructs optimized for bright fluorescence in plant cells (mGFP5;

generously provided by J. Haseloff [MRC, Cambridge, England]) were engineered to localize the GFP signal to various intracellular compartments. The mGFP5 localized to the ER contained a pathogenesis-related signal sequence (SS) and H(K)DEL ER retention signal inserted into pUCAP (a pUC19-based vector generously provided by R. Sederoff [North Carolina State University]) behind the cauliflower mosaic virus (CaMV) 35S promoter. For cytosolic localization, the gene encoding soluble mGFP5 was amplified by polymerase chain reaction (PCR) without the SS or HDEL retention sequence and with restriction enzyme sites appropriate for insertion into the pUCAP behind a 35S promoter. The cell-wall construct was made by PCR amplification of GFP without the HDEL retention sequence but containing the SS and appropriate restriction enzyme sites. The absence of the HDEL retention sequence allowed for the mGFP5 to follow the “default” protein secretion pathway described by Denecke and colleagues (6). This default pathway facilitates secretion of proteins to the cell wall independent of active sorting mechanisms.



**Figure 1. Transient expression of mGFP5 in onion epidermal cells.** Six separate epidermal peels were bombarded with mGFP5 localized to the ER. The number of cells per peel containing GFP fluorescence was counted every 2 h. Expression of mGFP5 first appears 8 h after bombardment and is maximal between 18 and 24 h after bombardment. Each bar represents an average of 6 onion peels. Error bars = standard error of the mean.

## Transformation

In separate experiments, 10  $\mu\text{g}$  of DNA encoding mGFP5 localized to the ER, the cell wall and the cytosol were delivered into onion epidermal cells using gold particle bombardment. Gold particles (1.0  $\mu\text{m}$ ; Bio-Rad, Hercules, CA, USA) were coated with DNA according to the manufacturer's directions. Alternatively, less expensive tungsten particles can be used (M. Kinkema, personal communication). Particles were bombarded into the onion epidermal cells using a Biolistic PDS-1000/He system (Bio-Rad) with 1100 psi rupture discs under a vacuum of 28 in Hg. After bombardment, the cells were allowed to recover for 18–22 h on agar plates at 22°C in continual light. For comparison of the ER-localized mGFP5 to other staining methods, onion peels were stained using 0.5  $\mu\text{M}$  rhodamine-6-g-

chloride (Molecular Probes, Eugene, OR, USA) for 20 min and then rinsed for 15 min in distilled water.

Wild-type GFP has a pK of 8.1 and is unstable at low pH (10). Therefore, GFP has not previously been seen in the cell wall, which has a pH of 5.0–6.0 (8,25). To visualize GFP in the cell wall, those cells bombarded with the cell-wall-localized mGFP5 were bathed in 20 mM piperazine-*N,N'*-bis (2-ethanesulfonic acid) (PIPES)-KOH (pH 7.0) following the 22-h incubation period and observed using a fluorescence dissecting microscope. Then, the same peels were soaked in 20 mM MES-HCl (pH 5.7) overnight and evaluated for mGFP5 fluorescence in the cell wall.

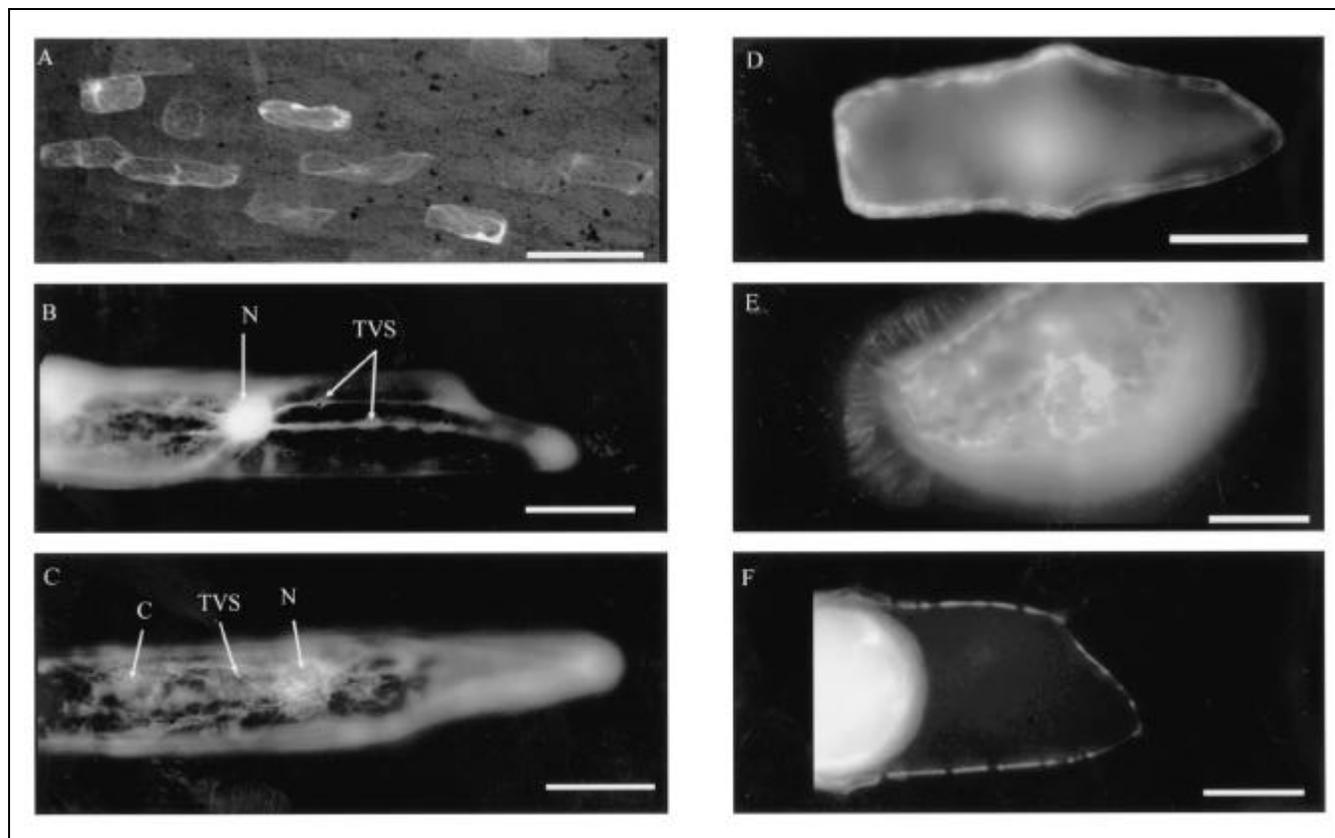
## Screening and Analysis

Peels were screened for mGFP5 fluorescence using a Leica MZ12 dissect-

ing microscope with a fluorescence attachment (Leica, Deerfield, IL, USA). Cells having fluorescence as well as normal cytoplasmic streaming were selected for further evaluation. Fluorescence and DIC microscopy were performed using Zeiss Axiovert® and Axiophot® microscopes equipped with 25 $\times$  and 40 $\times$  water, and 100 $\times$  oil immersion lenses (Carl Zeiss, Thornwood, NY, USA). Images were captured using a Pentamax Cooled CCD Camera (Model TE/CCD-K1317; Princeton Instruments, Princeton, NJ, USA) and analyzed using MetaMorph™ and Image One™ software (Universal Imaging, West Chester, PA, USA).

## RESULTS

To determine the utility of epidermal peel monolayers for imaging recombi-



**Figure 2. Localization of mGFP5 in epidermal onion cells.** (A) High transformation rates are seen in this overview of onion cells 18 h after mGFP5-gold-particle bombardment. mGFP5-ER expression is shown. Scale bar = 500  $\mu\text{m}$ . (B) Soluble GFP appears in the cytoplasm, nucleus, N, and in the transvacuolar strands, TVS, which contain cytoplasm. (C) GFP localized to the ER surrounds the nucleus N, and fluoresces in the cortical ER, C and the TVS. (D) GFP localized to the cell wall. (E) Cell-wall-localized GFP also appears in the ER. Plasmolysis with 1 M NaCl shows that GFP fluorescence is also within the Hechtian threads. GFP fluorescence is absent in the cell wall at normal cell wall pH. (F) Cell-wall-localized GFP fluoresces in the cell wall when bathed in buffer (pH 7.0) for 12 h. Scale bars in Panels B–F = 50  $\mu\text{m}$ .

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nant forms of mGFP5, we utilized the ability of these cells to transiently express mGFP5 through particle bombardment. To determine the efficiency of this method, six separate peels were evaluated for mGFP5 fluorescence every 2 h. Bombardment resulted in transformation of 60–100 cells per epidermal peel (2%–5%). GFP fluorescence was observed as early as 8 h after bombardment, with maximum fluorescence occurring between 18–22 h (Figure 1). If stored at 4°C after 22 h of incubation at 22°C, the peels were usable for up to 10 days post-bombardment. Fluorescence in transformed cells was about 720-fold greater than in bombarded, non-transformed cells. Both non-bombarded cells and bombarded, non-transformed cells showed little to no autofluorescence at 540/50 nm. Bombarded, non-transformed cells were identified as those cells that contained gold particles (as seen in brightfield), but had no GFP fluorescence. These cells provided a control to test for deleterious effects of the bombarded construct. Cytoplasmic streaming was used as an indicator of

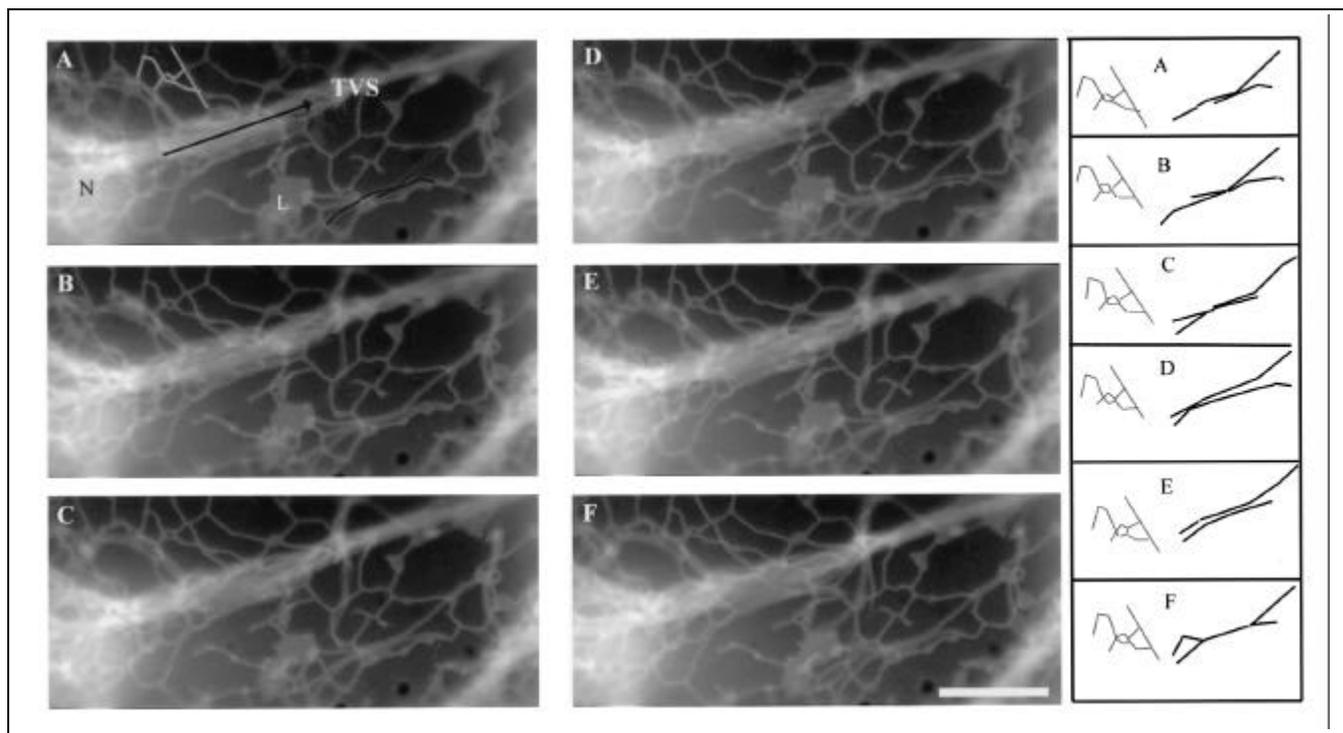
cell viability. Visual, qualitative comparison of streaming in transformed cells to that of adjacent non-transformed cells showed that the GFP constructs had no deleterious effects on the onion cells (data not shown).

Onion cells transiently expressing various localized GFP constructs could be imaged at a theoretical resolution of approximately 200 nm, permitting the observation of organelles and other structures at the subcellular level. GFP localized to the ER, the cytoplasm and the cell wall showed distinct patterns of fluorescence (Figure 2). With GFP localized to the ER, it was possible to clearly image tubules and lamellar regions in the cortical ER, and the ER present in transvacuolar strands. ER was also seen surrounding the nucleus. The dynamics of the ER were recorded and are shown in Figure 3 (see also, <http://www4.ncsu.edu/unity/users/e/evbrown/www/allen.html>). Small rapidly moving particles in or on the ER, first observed in *Acetabularia* extracts by Allen and Schumm (3), are clearly seen using this technique. ER-

localized GFP was compared to cells stained with DiOC<sub>6</sub>(3) and rhodamine-6-g-chloride. These stains show similar patterns to the GFP; however, they also stained other organelles in a nonspecific manner, such as the staining of mitochondria (data not shown).

The soluble GFP showed a pattern different from the GFP localized to the ER. The cytoplasmic GFP lacked the reticulate pattern seen with the ER construct and was also localized in the nucleus because of its small molecular weight (27 kDa). Active cytoplasmic streaming was observed under fluorescence microscopy with this construct.

mGFP5 lacking the HDEL sequence did not fluoresce in the cell wall when peels were incubated on agar medium maintained at pH 5.7, although fluorescence was seen in the ER/Golgi complex. When peels were bathed overnight in buffer at pH 7.0, fluorescence appeared in the cell wall as well. Fluorescence first appeared 4 h after buffer application. Plasmolysis with 1 M NaCl showed that staining does occur in the cell wall (Figure 2) and that GFP



**Figure 3. Dynamics of GFP localized to the ER.** mGFP5 localized in the ER is used to visualize ER dynamics within epidermal cells of onion. Panels A–F are time-lapse images taken 2 s apart. The green line-drawings highlight strands within the cortical ER that remain stationary. The red line-drawings highlight those strands in the cortical ER, which have subtle movements over time. The ER within the transvacuolar strands moves rapidly (arrow). See <http://www4.ncsu.edu/unity/users/e/evbrown/www/allen.html> for better visualization of the ER within the TVS. These images illustrate the ER around the nucleus, N, in the transvacuolar strands, TVS, and the cortical ER that contains strands and lamellar sheets, L. Scale bar in Panel F = 10  $\mu$ m.

was present in the Hechtian threads. This indicated the presence of vesicles containing GFP in these threads. Cell wall fluorescence was subsequently quenched by soaking overnight in a buffer at pH 5.7 (data not shown).

## DISCUSSION

Cells from the onion epidermis have facilitated the study of cell structure and have demonstrated their suitability for light microscopy. We have taken advantage of these properties and utilized them for the study of organelle structure and dynamics in vivo using transient GFP expression methods.

The epidermal peel from *Allium cepa* has many advantages for imaging cellular structures. These cells are large, transparent, have little autofluorescence and are present in a single layer, making them ideal for observing GFP fusion proteins. This expression method utilizes the advantages that GFP has over commonly used staining procedures. GFP fluorescence is independent of substrate availability and is devoid of diffusion complications found with other staining methods. Localized GFP is more specific than stains that might have nonspecific staining patterns, such as that seen with DiOC<sub>6</sub>(3) and rhodamine-6-g-chloride. Imaging over long time periods is also possible because of the stability of GFP. For example, cytoplasmic streaming over long time periods and nuclear migration (which can take several hours) can be studied following introduction of GFP with specific signal and targeting sequences. Another very important use will be the bombardment of onion cell monolayers for testing GFP fusion proteins before stable transformation is attempted.

Whereas the advantages are numerous, the potential drawbacks of this system are minimal. The cells in the epidermal peel are subject to wounding during the isolation procedure; however, it is easy to assess the health of the cells by the presence of cytoplasmic streaming. Also, it is necessary to consider the choice of promoters. For these experiments, we used the CaMV 35S promoter. We were unable to detect GFP fluorescence using the *Arabidop-*

*sis* heat-shock promoter in onion epidermal cells. This may be because onion is a monocot or because the tissue used is the epidermal layer. These were the only two promoters we tested, and further manipulation of the experimental promoter could increase the number of effective promoters.

A drawback of GFP fluorescence in a multicellular tissue is that quantification is difficult. However, because epidermal peels are one-cell thick, out-of-focus fluorescence from adjacent cells does not complicate the analysis of fluorescence intensity. This type of analysis will be very useful for studying physiological events and intra- or intercellular communication in living cells.

Until now, to our knowledge, GFP has not been seen in the cell wall. The lack of visualization is attributed to the low pH of the cell wall. GFP fluorescence is pH-dependent with fluorescence intensity decreasing at low pH (6,30). At a pH of 6.0, the fluorescence intensity of GFP is 50% (22). The cell-wall pH was changed by soaking the onion peel in buffer (pH 7.0). Following this treatment, the GFP fluorescence was observed in the cell wall. GFP fluorescence in the cell wall appeared 4 h after the wall was buffered at a pH of 7.0, suggesting that newly synthesized GFP was secreted into the cell wall. The development of the GFP fluorophore takes approximately 4 h (11,12). If GFP present in the cell wall before buffering had an intact fluorophore, but lacked the ability to reach full fluorescence intensity due to the normal cell-wall pH, fluorescence would appear quickly once the buffer (pH 7.0) was supplied. GFP fluorescence was seen in the Hechtian threads following plasmolysis, when peels were buffered at pH 7.0 or pH 5.7. This suggests that under normal cell-wall pH, the fluorophore is unstable, hence quenching the fluorescence. Under neutral pH conditions, the fluorophore is stable, thus fluorescence was detected.

In comparison to maintenance of cell cultures and the development of stable transformants, the methods described in this paper are simple, inexpensive and rapid, therefore, making this a powerful experimental tool. The ability to use onion epidermal cells to transiently express GFP is a method

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that will allow for *in vivo* studies of physiological events and provide a test specimen for localizing the expression of gene fusions.

## REFERENCES

1. Allen, N. and D. Brown. 1988. Dynamics of the endoplasmic reticulum in living onion cells in relation to microtubules, microfilaments and intracellular particle movement. *Cell Motil. Cytoskeleton* 10:153-163.
2. Allen, G., G. Hall, L. Childs, A. Weissinger, S. Spiker and W. Thompson. 1993. Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. *Plant Cell* 5:603-613.
3. Allen, N. and J. Schumm. 1990. Endoplasmic reticulum, calciosomes and their possible roles in signal transduction. *Protoplasma* 154:172-178.
4. Amstel, A. 1996. A relationship between callose and ectodesmata in epidermal cells of *Allium cepa* L. *Plant Cell Rep.* 15:707-711.
5. Boevink, P., S. Santa Cruz, C. Hawes, N. Harris and K. Oparka. 1996. Virus-mediated delivery of the green fluorescent protein to the endoplasmic reticulum of plant cells. *Plant J.* 10:935-941.
6. Denecke, J., J. Botterman and R. Deblaere. 1990. Protein secretion in plant cells can occur via a default pathway. *Plant Cell* 2:51-59.
7. Ding, J., P. Badot and B. Pickard. 1993. Aluminium and hydrogen ions inhibit a mechanosensory calcium-selective cation channel. *Aust. J. Plant Physiol.* 20:771-778.
8. Felle, H. 1998. The apoplasmic pH of *Zea mays* root cortex as measured with pH-sensitive microelectrodes: aspects of regulation. *J. Exp. Bot.* 49:987-995.
9. Grebenok, R., G. Lambert and D. Galbraith. 1997. Characterization of the targeted nuclear accumulation of GFP within the cells of transgenic plants. *Plant J.* 12:685-696.
10. Haseloff, J., K. Siemering, D. Prasher and S. Hodge. 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci. USA* 94:2122-2127.
11. Heim, R., A. Cubitt and R. Tsien. 1995. Improved green fluorescence. *Nature* 373:663-664.
12. Heim, R., D. Prasher and R. Tsien. 1994. Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* 91:12501-12504.
13. Köhler, R. 1998. GFP for *in vivo* imaging of subcellular structures in plant cells. *Trends Plant Sci.* 3:317-320.
14. Köhler, R., W. Zipfel, W. Webb and M. Hanson. 1997. The green fluorescent protein as a marker to visualize plant mitochondria *in vivo*. *Plant J.* 11:613-621.
15. Lichtscheidl, L. and L. Foissner. 1996. Video microscopy of dynamic plant cell organelles: principles of the technique and practical application. *J. Microsc.* 181:117-129.
16. Lichtscheidl, L., S. Lancelle and P. Hepler. 1990. Actin-endoplasmic reticulum complexes in *Drosera*: their structural relationship with the plasmalemma, nucleus, and organelles in cells prepared by high pressure freezing. *Protoplasma* 155:116-126.
17. Lichtscheidl, I. and W. Url. 1990. Organization and dynamics of cortical endoplasmic reticulum in inner epidermal cells of onion bulb scales. *Protoplasma* 157:203-215.
18. Mansour, M. 1995. NaCl alteration of plasma membrane of *Allium cepa* epidermal cells: alleviation by calcium. *Plant Physiol.* 145:726-730.
19. Miesenböck, G., D. De Angelis and J. Rothman. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394:192-195.
20. Oparka, K., D. Prior and J. Crawford. 1994. Behaviour of plasma membrane, cortical ER and plasmodesmata during plasmolysis of onion epidermal cells. *Plant Cell Environ.* 17:163-171.
21. Oparka, K., D. Prior and N. Harris. 1990. Osmotic induction of fluid-phase endocytosis in onion epidermal cells. *Planta* 180:555-561.
22. Patterson, G., S. Knobel, W. Sharif, S. Kain and D. Piston. 1997. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* 73:2782-2790.
23. Pont-Lezica, R., J. McNally and B. Pickard. 1993. Wall-to-membrane linkers in onion epidermis: some hypotheses. *Plant Cell Environ.* 16:111-123.
24. Presley, J., C. Nelson, T. Schroer, K. Hirschberg, K. Zaal and J. Lippincott-Schwartz. 1997. ER-to-golgi transport visualized in living cells. *Nature* 389:81-85.
25. Putnam, R. 1998. Intracellular pH regulation, p. 293-305. *In* N. Sperelakis (Ed.), *Cell Physiology Source Book*. Academic Press, San Diego.
26. Quader, H. and H. Fast. 1990. Influence of cytosolic pH changes on the organization of the endoplasmic reticulum in epidermal cells of onion bulb scales: acidification by loading with weak organic acids. *Protoplasma* 157:216-224.
27. Quader, H., A. Hofmann and E. Schnepf. 1987. Shape and movement of the endoplasmic reticulum in onion bulb epidermis cells: possible involvement of actin. *Eur. J. Cell Biol.* 44:17-26.
28. Quader, H. and E. Schnepf. 1986. Endoplasmic reticulum and cytoplasmic streaming: fluorescence microscopical observations in adaxial epidermis cells of onion bulb scales. *Protoplasma* 131:250-252.
29. Runthala, P. and S. Bhattacharya. 1991. Effect of magnetic field on the living cells of *Allium cepa* L. *Cytologia* 56:63-72.
30. Sansebastiano, G., N. Paris, S. Marc-Martin and J. Neuhaus. 1998. Specific accumulation of GFP in a non-vacuolar compartment via a C-terminal propeptide-mediated sorting pathway. *Plant J.* 15:449-457.
31. Sheen, J., S. Hwang, Y. Niwa, H. Kobayashi and D. Galbraith. 1995. Green-fluorescent protein as a new vital marker in plant cells. *Plant J.* 8:777-784.
32. Sonobe, S. and H. Shibaoka. 1989. Cortical fine actin filaments in higher plant cells visualized by rhodamine-phalloidin after pretreatment with m-maleimidobenzoyl N-hydroxy-succinimide ester. *Protoplasma* 148:80-86.
33. Wincur, A., G. Zhang and L. Staehelin. 1998. Auxin deprivation induces synchronous golgi differentiation in suspension-cultured tobacco BY-2 cells. *Plant Physiol.* 117:501-513.

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