Development of a Cytosolic pH Reporter for Tobacco By2 Cells

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Development of a Cytosolic pH Reporter for Tobacco BY2 Cells

A Thesis Presented

by

MICHAEL EDWARD URBANOWSKI

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Graduate Program in Plant Biology
Development of a Cytosolic pH Reporter for Tobacco BY2 Cells

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DEDICATION

To Dr. Robert I. Bertin,

who gave me my first microscope,

and especially to my parents,

Ed and Jean

for giving me the objective that I was missing.
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ABSTRACT
DEVELOPMENT OF A CYTOSOLIC PH REPORTER FOR TOBACCO BY-2 CELLS
SEPTEMBER 2012
MICHAEL EDWARD URBANOWSKI, B.S., UNIVERSITY OF MASSACHUSETTS
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The regulation of pH is a critical homeostatic function of plant cells. In addition to acting as the primary cationic species responsible for energizing the plasma membrane, protons likely act as an important regulator and messenger. Despite this importance, few studies have thoroughly described cytosolic pH patterns as the plant cell progresses through the cell cycle. To investigate pH in plant cells, I chose *Nicotiana tabacum* (tobacco) Bright Yellow-2 (BY-2) cells as a model system. My research has two aims. First, I will measure and report the interphase cytosolic pH of BY-2 cells. Next, I will assay the cytosolic pH as BY-2 cells progress through mitosis and cytokinesis. I hypothesize that pH patterns are be temporally or spatially associated with structures such as the mitotic spindle or the phragmoplast. To investigate cytosolic pH in BY-2 cells, I will develop a cytosolic pH reporter based on a pH sensitive ratiometric fluorescent dye. This dye will be able to resolve both temporal and spatial changes in pH throughout the cytosol while imposing a minimal amount of stress on BY-2 cells. I found that pH-GFP, a variant of eGFP, had qualities of a robust pH reporter. To introduce the dye, explored biolistic bombardment, *Agrobacterium* mediated transient transformation, and polyethylene glycol mediated transformation as methods for introducing the pH-GFP gene into BY-2 cells. I observed very few transformation events using these methods and my observations did not support these approaches as suitable for introducing pH-GFP into BY-2 cells.
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CHAPTER 1
INTRODUCTION

1.1 Regulation of pH is a necessary and universal function of cells

The molecular machinery of the cell functions in an aqueous environment. Water molecules, themselves, are critical participants in many of the chemical reactions that occur between cellular constituents. However, the requirement for hydration alone is not sufficient to guarantee a hospitable reaction environment. Rather, the properties that affect the reactivity of water and aqueous substrates must also be tightly controlled within the cell. Chief among these parameters is the concentration of hydrogen ions, the pH of the cytosol.

Like water, hydrogen ions are popular participants in organic reactions. Most cellular polymers contain acidic or basic residues, making their conformations (and reactivity) sensitive to the availability of protons (Swanson et al., 2011). This implies that cells carry out pH homeostasis to maintain reaction-favorable internal conditions despite unfavorable extracellular conditions. To support this assumption, when the extracellular environment around bacteria was acidified, the bacteria coped with this acidification by restoring their cytosolic pH to near pre-treatment values (Martinez et al., 2012). Similar evidence for homeostatic pH maintenance is also observed in mammalian cells (Casey et al., 2010), yeast (Orij et al., 2011) and algal cells (Bethmann and Schoenknecht, 2009).

Bacteria, fungi, and plants energize their plasma membrane with a proton gradient and for this reason pH homeostasis is particularly important in these groups. Energizing the membrane involves coupling ATP hydrolysis to proton export at the plasma membrane by means of H⁺-ATPases. The resulting proton gradients are harnessed to transport ions and metabolites such as phosphate, nitrate, potassium, sodium, sugars, amino acids, and peptides into cells (Taiz and Zeiger, 2006; Sano et al., 2009). Uptake of these organic and inorganic nutrients is essential for cell growth (Bassil et al., 2011; Haruta and Sussman, 2012).
1.2 Mechanisms of pH homeostasis

Proton pumps are not the only mechanism for mediating intracellular pH. Felle and colleagues (2001) reviewed three distinct mechanisms for cytosolic pH regulation.

Immediate protection of the cytosol against sudden pH changes is accomplished by a strong buffering system founded on soluble weak acids. Ultimately, however, protons must be removed from this buffering system so that it maintains responsiveness to future changes. Two additional mechanisms mediate shuttling of protons from the cytosol and the buffering system. First, the aforementioned plasma membrane H\(^+\)-ATPase and vacuolar H\(^+\)-ATPases (in plants) actively move protons to the extracellular environment and to an acidic sink in the vacuole, respectively. Second, protons act as products and reactants in numerous metabolic reactions. As a result, free protons are removed or delivered to the cytosol based on the metabolic activities of the cell.

The presence of strong homeostatic mechanisms for pH regulation has importance for observed sub-cellular pH patterns, such as alkaline or acidic domains. This is that any observed change to the pH homeostasis, compared to a steady-state cell, must result from an active process; either a change in the pH regulatory machinery or a process that directly competes with the action of pH regulatory machinery.

1.3 Protons in plant cells: non-traditional messengers and regulators

Intriguingly, proton homeostasis in plants is balanced by the use of protons in messenger and regulatory roles. Felle and colleagues (2001) described protons as a unique type of messenger and regulator. Contrasting the intracellular nature of protons to another well described cationic secondary messenger, calcium, the authors point out that protons both diffuse faster and have less specificity for downstream targets than calcium. Because protons can have dramatic influence over the conformation of many cellular polymers, specificity exists primarily with the downstream target.
Cytosolic pH changes are regulators of plant cell stress responses. One of the most profound examples of pH patterning in cellular regulation was described by Tournaire-Roux and colleagues (2003). It was previously observed that conduction of water through the plant vasculature ceases when the soil medium was saturated with water. However, the link between soil flooding and decreased vascular activity was not understood. The team uncovered a motif on aquaporin proteins that was sensitive to cytosolic pH. Upon cytosolic acidification, driven by the anoxic conditions of the saturated soil, the aquaporins showed diminished activity. Furthermore, other examples illustrate the importance of cytosolic pH changes as a response to stress. Cytosolic acidification was found to be a marker of programmed cell death in plants (Young et al., 2010).

Changes in proton distribution may help change the dynamics of the cell wall. In response to mechanical stimulation, *Arabidopsis thaliana* roots underwent a localized increase in the concentration of cytosolic calcium at the site of the mechanical perturbation which was followed by cytosolic alkalization and corresponding apoplastic acidification (Monshausen et al., 2009). Following these responses, the root exhibited an avoidance response to the perturbation. In another example, a growth-dependent acidic tip and sub-apical alkaline band was observed in pollen tubes of *Lilium longiflorum* (Feijo et al., 1999; Lovy-Wheeler et al., 2006). This pH pattern may be due to the regulated exclusion of a H⁺-ATPase from the apical membrane domain (Certal et al., 2008). The role of the apical acidic domain and the sub-apical alkaline fringe in pollen tubes is less-well understood. One hypothesis for the existence of the acidic tip and alkaline fringe is to regulate the apical actin cytoskeleton by modulating the activity of actin-binding proteins (Lovy-Wheeler et al., 2006).
1.4 Measuring cytosolic pH in plant cells

Despite tremendous advances in molecular biology and biochemistry during the past thirty years, many fundamental questions about plant cell pH physiology remain unresolved. Kurkdjian and Guern (1989) published a thorough review of several classic techniques for measuring cytosolic pH in vivo: NMR spectrometry, acid-loading and chemical probe measurements, and H⁺-selective microelectrode measurements. Additionally, pH sensitive fluorescent dyes are recently popular as cellular pH reporters and their use is reviewed by Swanson and colleagues (2011).

NMR spectrometry based on phosphorus resonances is a powerful tool for studying cytosolic pH and was applied by Tournaire-Roux and colleagues for their studies in pH mediated aquaporin gating (2003). The technique measures the spectral shift associated with inorganic phosphorus. As the cytosolic proton environment around the phosphorus atoms changes, then the peaks corresponding to phosphorus atoms shift. The use of $^{31}$P NMR to observe cytosolic pH has several drawbacks. A large mass of sample cells must be present and the technique reports inorganic phosphorous shifts corresponding to an average proton environment. As a result, this technique is inadequate for studying subcellular pH patterning. Finally, the technique has poor temporal resolution so that events during the mitotic timeline cannot be resolved (Kurkdjian and Guern, 1989).

Acid-loading and chemical probe measurements take advantage of weak acids whose non-dissociated form is membrane permeable. The non-dissociated form of the acid diffuses down its concentration gradient across the cell membrane and into the cytoplasm. The pools of weak acid on both sides of the plasma membrane dissociate according to the pH of their respective environments. A chemical probe then measures the relative proportion of dissociated and non-dissociated acid on either side of the plasma membrane. These measurements, combine with the known pKa of the weak acid, can be used to calculate the cytosolic pH (Guern et al., 1991). Several drawbacks exist for
this technique. First, the calculations assume that only the non-dissociated form of the weak-acid diffuses across the membrane. Second, the calculations require a difficult estimation of the volume of the cytoplasm. Third, the technique is insensitive to sudden pH changes. Fourth, the technique cannot resolve spatial differences in pH such as subcellular domains. Fifth, the cell might be traumatized by the placement of a cytosolic chemical probe. Finally, large concentrations of the weak-acid load will buffer and change the physiological pH of the cell system (Kurkdjian and Guern, 1989).

The H$^+$-selective microelectrode is another tool for measuring cytosolic pH. Similar to the chemical-probe technique, microelectrode measurements require possibly traumatic invasion of the cell wall and plasma membrane with a microelectrode probe that is sensitive to hydrogen ions. Once inside the cell, the concentration of hydrogen ions surrounding the probe is measured by estimating the potential difference across two selectively permeable membranes, one of which permits the movement of protons and the other which acts as a reference. In addition to being an invasive technique, this technique cannot resolve spatial pH patterns (Kurkdjian and Guern, 1989).

Recently, fluorescent dyes have become popular and powerful reporters of intracellular pH (Swanson et al., 2011). Fluorescent dyes sensitive to pH can be grouped into two categories based on their molecular identity; protein-based dyes and non-protein based dyes. Non-protein based dyes, such as pH sensitive 2’,7’-bis(carboxyethyl)-5-(and-6)-carboxyfluoresceine (BCECF) must be introduced into plant cells by microinjection (Lovy-Wheeler et al., 2006; Cardenas et al., 2008) or bombardment (Bothwell et al., 2006). BCECF is also an example of a special class of fluorescent dyes which are amenable to ratiometric fluorescence imaging (Fig. 1; Swanson et al., 2011).

Ratiometric fluorescence imaging is a technique that can minimize the spatial and temporal effects on the dye probe due to properties of the cytosol other than proton concentration. In ratiometric fluorescence imaging, excitation of a fluorescent dye at a specific wavelength produces a signal that is dependent on a property of interest, such as
the proton concentration. Excitation at a second wavelength produces an emission signal that is independent of the cytosolic property under investigation (Fig. 3). When the ratio of the emission signals from the two excitations is taken, the quotient is dependent on the property of interest, but independent of other confounding properties, such as path-length or photobleaching (Bright et al., 1989). BCECF has intrinsic fluorescence properties that allow for dual excitations, one of which produces a pH dependent emission signal, and the other which produces a reference signal (Fig. 2; Fig. 3; Graber et al., 1986).

Protein-based dyes are advantageous because their genes can be cloned and transformed into plant cells. After an initial transformation event, transgenic plant cells can be selected for, using antibiotics, until stable lines are created (Michard et al., 2008; Martinez et al., 2012). In this case, the trauma of microinjection or bombardment is traded for the possibility of causing a physiologically consequential lesion in the genome. Modified GFP molecules are widely used as pH reporters and several variations exist. pHluorin was the first published use of a modified-GFP for pH reporting (Moseyko and Feldman, 2001). Another GFP variant, H148D/pH-GFP (pH-GFP) has also been characterized and used for cytosolic pH reporting (Fasano et al., 2001). Similar to BCECF, both pHluorin and pH-GFP are amenable to ratiometric fluorescence imaging and have dynamic reporting ranges that encompass the expected cytosolic pH range.

The use of pH sensitive fluorescent dyes has a number of advantages over $^{31}$P NMR spectrometry, acid-load/chemical probe, and microelectrode techniques. With
**Figure 1.** The molecular structure of BCECF that predominates in neutral solution (Invitrogen, 2006).

**Figure 2.** Predicted structure of eGFP. A mutation from histidine to aspartic acid (white arrow) yields pH-GFP (Elsliger et al. 1999). Figure made by Cn3D program (Wang et al. 2000)
fluorescence imaging, spatial pH changes can be monitored within a single cell. This allows the observer to view subcellular pH domains. Fluorescence imaging also allows the observation of sudden changes in pH to the limits of the exposure time and shutter speed for the imaging apparatus, dramatically improving the temporal resolution over other methods. However, the use of pH sensitive fluorescent dyes presents unique challenges. Feijo and colleagues (1999) observed that when the concentration of dye was high, then pollen tubes of *Lilium longiflorum* stopped growing, indicating a stress response. The authors further reviewed that pH dyes, themselves, act as weak acids with their own pKa values and hypothesized that, at high concentrations, the dyes act as cytosolic buffers to dissipate the apical pH gradient in pollen tubes. Conversely, the group also discussed a necessary lower-limit to the dye concentration. If the dye concentration is too low, then there is not enough dye signal to provide contrast.

The steady-state cytosolic pH to the plant-cell cytosol traditionally assumed to be close to neutral. However, despite the diversity of techniques available for studying intracellular pH, no consensus value has been adopted.

1.5 Development of a cytosolic pH reporter

As described in the introduction, several different pH sensing techniques are popular for measuring cytosolic pH in plant cells. My questions ask about spatial and temporal pH patterning. Therefore, I will use a fluorescent probe to survey the cytosolic proton environment because fluorescent probes can report pH with good spatial and temporal resolution.

1.5.1 Cytosolic pH patterns throughout the plant cell cycle

Despite evidence demonstrating that pH regulation is important for plant cell physiology, few studies have examined the plant cell cytosol for evidence subcellular pH patterns throughout the cell cycle (Roos, 1992). My work investigates two questions
about pH regulation in the plant cell cytosol. First, I asked what the steady-state pH of the plant cell cytosol is during interphase. Although it is possible that interphase pH patterning is different based on species, I chose a popular plant-cell culture system to investigate this phenomenon. Second, I asked whether the pH of the cytosol changes as the plant cell progresses through mitosis and cytokinesis. I identified these landmarks in the cell cycle as targets for pH patterning because highly organized mitotic and cytokinetic machinery is constructed and remodeled continually during these transitions. I hypothesize that changes in subcellular pH patterning from an interphase pH baseline participate in the construction and remodeling of this machinery. To investigate these questions, I explored several approaches toward developing a robust reporter of subcellular cytosolic pH.

1.5.2 Choice of plant-cell system

The choice of cell system has important ramifications for the design of a pH reporter. For instance, *A. thaliana* plants are amenable to transformation by *Agrobacterium tumefaciens* mediated floral dip (Clough and Bent, 1998). For this system, a protein-based pH reporter transgene could be introduced into *A. thaliana* under the control of a tissue-specific promoter. In contrast, *Lilium longiflorum* is not amenable to transformation, but has been studied using microinjection and bombardment of dyes.

I will use tobacco Bright Yellow - 2 (BY-2) cells for the development of a pH reporter system. BY-2 cells are a well-studied plant-cell physiology model and are often referred to as the HeLa cells of plant biology (Nagata et al., 2004) These cells grow in homogenous liquid culture at room temperature. They are easily visualized using light microscopy because BY-2 cells in liquid culture are large and grow in filaments, in contrast to the liquid culture aggregates observed in many other plant-cell liquid cultures. Furthermore, BY-2 cells are used extensively for plant-cell cycle studies, hormonal control studies, biochemistry, and molecular biology. A diverse set of protocols,
including cell-cycle synchronization, has been well-established for this cell system and are reviewed in two books (Nagata, Lörz, and Widholm, 2004, 2006).

1.5.3 Choice of pH reporter and introduction method

Because BY-2 cells are amenable to both physiological and molecular manipulation, both non-protein based and protein based pH reporter dyes could be used as a cytosolic pH reporter. To determine the best pH reporter for this cell-system, I describe several characteristics of an ideal cytosolic pH reporter. First, it should be able to be introduced in a way that minimizes damage to the cell. Second, the technique should be repeatable. Third, the reporter should be introduced so that the concentration of dye does not stress the cell. Fourth, the reporter should be able to go into many cells, so that the cell population can be synchronized for mitotic studies.

First, I will attempt to measure pH in BY-2 cells by microinjecting BCECF conjugated to a 70 kDa dextran molecule (BCECF-dextran). Microinjection is a well-established technique for cell-physiology studies. Furthermore, BCECF is amenable to ratiometric imaging and has been previously used for studies in plant-cell pH physiology (Fig. 3; Feijo et al., 1999; Scott and Allen, 1999; Halperin et al., 2003). Conjugation of a 70 kDa dextran to the BCECF molecule minimizes leakage of the reporter dye into the acidic vacuole, which could confuse how the dye reports cytosolic pH. Although invasive and potentially traumatic to the cell, the technique could yield results quickly. I also have a microinjection apparatus available for use in the Hepler laboratory.

If microinjection is not found to be a robust technique for introducing BCECF-dextran, I will alternatively try to introduce BCECF-dextran into BY-2 cells by
Figure 3. Fluorescence emission as a function of excitation wavelength for (A) eGFP, (B) pH-GFP, (C) BCECF, (D) the equation that relates hydrogen ion concentration to a fluorescence ratio for a given pH reporter. Note that the intensity with excitation at 440 nm is ~pH independent. A-B is redrawn from Elsliger et al. (1999). C is redrawn from Invitrogen. D, $K_a$ is the acid dissociation constant for the dye. R is the ratio of emission fluorescence at the dependent and independent excitation wavelengths. $R_A$ and $R_B$ are based on the calibration of the dye and represent acid and basic endpoints of the weak-acid dye titration. Finally, $F_A$ and $F_B$ represent the fluorescence intensities following excitation at the independent wavelength for both the acidic and basic extremes of the dye range.
microprojectile bombardment. Although microprojectile bombardment is traditionally a technique used for introducing transgenes into plant cells, Bothwell and colleagues (2006) found that non-protein fluorescent dyes could also be introduced into plant cells by microprojectile bombardment.

If the placement of BCECF-dextran into the BY-2 cytosol is not supported as a robust indicator of cytosolic pH, I will then explore the use of a protein-based pH reporter. I have obtained a pH-sensitive GFP variant from the Gilroy Laboratory (Fasano et al., 2001). The GFP molecule is a further modification of enhanced GFP (S65T) to allow the aqueous environment access to the fluorophore, increasing both the sensitivity of the variant to pH changes and raising the pKa of the GFP variant from 6.0 to 7.8. These modifications were created by a lesion in the S65T GFP gene to affect a switch in the histidine residue at the 148\textsuperscript{th} amino acid position to aspartic acid. This change resulted in the creation of S65T/H148D (pH-GFP) (Elsliger et al., 1999).

The pH-GFP can be introduced into BY-2 cells through several different methods. I will first attempt to introduce pH-GFP into BY-2 cells by microprojectile bombardment of DNA containing the gene for pH-GFP under the control of 35S promoter. Microprojectile bombardment can be quickly carried out to yield transiently transformed cells (Morikawa et al., 1989; Iida et al., 1990). However, microprojectile bombardment might be traumatic to BY-2 cells and involves entry of a gold or tungsten microprojectile into the BY-2 cell cytosol (Russell et al., 1992).

As an alternative to microprojectile bombardment of pH-GFP, BY-2 cells can be stably transformed using Agrobacterium tumefaciens. A. tumefaciens is a gram-negative soil dwelling bacteria. To accomplish conjugation, the bacteria carry a Ti plasmid. Encoded on this plasmid are the genes necessary to form a type IV-like conjugating apparatus. Additionally, the Ti plasmid carries genes for site-specific recombinases that excise virulence genes. These virulence genes are also harbored on the Ti plasmid. Chaperone proteins mediate the movement of these genes through the conjugation
apparatus. Once inside the plant cell, a final set of Ti plasmid-encoded genes facilitates recombination of the virulence genes at random points in the plant-cell genome. To take advantage of *A. tumefaciens* as a tool for introducing trans-genes into plant cells, researches have disarmed the Ti plasmid by replacing the virulence genes with genes-of-interest. These genes will recombine into the plant-genome after the conjugal transfer of DNA.

Although a stable transformation mediated by *A. tumefaciens* is an attractive approach for introducing pH-GFP into BY-2 cells, it does have several drawbacks. First, it is a long process. Initial transformation can be accomplished within weeks, but the BY-2 cells must be placed under antibiotic selection for several months before creation of a stable line is accomplished. Second, a stable transformation of BY-2 cells is resource intensive. Dissimilar to bacteria or animal cells, plant cells cannot yet be cryogenically stored. As a result, stable lines of BY-2 cells must be continually sub-cultured to maintain the viability. To evade these drawbacks, it is possible instead to use *A. tumefaciens* to transiently transform BY-2 cells, similar to how microprojectile bombardment transiently introduces gene copies into cells. Buschmann and colleagues (2011) demonstrated that this was possible for their studies on the BY-2 cytoskeleton.

Buschmann and colleagues (2011) also showed evidence that the use of elicitors of conjugation and the identity of the *A. tumefaciens* strain used for the transient transformation protocol have a dramatic impact on the efficiency of *A. tumefaciens* mediated transient transformation. To further explore the possibilities for introducing the gene encoding pH-GFP into BY-2 cells, I will attempt to reproduce Buschmann and colleagues protocol for transient transformation. To maximize the efficiency of these transformations, I have obtained several strains of *A. tumefaciens*. These strains are GV3101 (Walker Laboratory, University of Massachusetts Amherst), EHA105 (Walker Laboratory, University of Massachusetts Amherst), and LBA4404 (Parkash Laboratory, University of Massachusetts Amherst). I will also test the transformation efficiency of
this protocol in the presence and absence of acetosyringone, a known elicitor of *A. tumefaciens* and plant-cell conjugation.

Finally, if I cannot repeat the work of Bushmann and colleagues (2011), then I will alternatively try polyethylene glycol (PEG) mediated transformation of BY-2 protoplasts. In this protocol, the cell wall is removed from the BY-2 cells and PEG is used to allow DNA to move through the plasma membrane. BY-2 cells show an ability to regenerate their cell walls after 24 hours following digestion and this protocol could yield a high frequency of transiently transformed BY-2 cells.

### 1.5.4 Calibrating the pH reporter dye

Although BCECF-dextran and pH-GFP report pH dependent ratios, the activity of the dye must be calibrated against a set of standards to measure the pH of the cytosol. These standards are solutions of pseudo-cytosol, buffered to known pH values. To calibrate our dye of choice, I will mix the dye into these solutions to establish a calibration curve. The linear constant of this calibration curve will allow me to relate the ratio reported by the reporter *in vivo* to the concentration of protons present in the cytosol according to Equation 1 (Figure 2).

BCECF-dextran is calibrated by introducing the BCECF-dextran dye into the pseudo-cytosol. In contrast, if pH-GFP is selected as a dye of choice of pH measurements, it must first be expressed in *E. coli* and subsequently purified. The purified pH-GFP will be introduced into the pseudo-cytosol mixture.

### 1.5.5 To quantify pH of the BY-2 cytosol

To quantify the interphase pH of the BY-2 cell cytosol, I will introduce the pH reporter of choice into BY-2 cells. I will then image cells during interphase using an inverted fluorescence light microscope. The ratio images will be processed using ImageJ software (available free: http://rsbweb.nih.gov/ij/index.html) and the average ratio values
for small regions of cytosol will be measured and reported. One powerful application of this pH reporter system is the ability to explore spatial and temporal changes in pH as the cell moves through the cell cycle. One of my goals is to use this pH reporter to look for pH domains around mitotic and cytokinetic machinery.
2.1 Measurement of cytosolic pH by microinjection of BCECF-dextran

I first approached cytosolic pH measurements by microinjecting 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) conjugated to a 70 kDa dextran molecule into the cytosol of tobacco BY-2 cells. Microinjection was chosen because it can be accomplished quickly, because a microinjection apparatus was already available, and because it has been shown to be a robust method for introducing molecules into plant cells.

Microinjection involves the placement of a small needle into the target cell. The needle carries the injectable chemical species. Pressure is supplied to the needle causing the chemical species to enter the cell through the tip of the needle. Likewise, BCECF is a well-characterized chemical dye with fluorescence properties suitable for intracellular ratiometric pH measurements (Invitrogen, 2006). Ratiometric imaging is ideal for pH measurements because the effects on the dye-reporter signal due to photo-bleaching and path-length are minimized (Bright et al., 1989). I used BCECF dye conjugated to a 70 kDa dextran molecule to prevent movement of the BCECF into the acidic plant cell vacuole.

On the day of microinjection, log-phase cells were plated onto glass coverslips coated with polylysine and suspended in BY-2 media. Polylysine forms ionic interactions with plant cell walls to immobilize the BY-2 cells during the microinjection process.

Since microinjection is a potentially traumatic process for living cells, cells were observed for 10 minutes following microinjection. Vigorous cytoplasmic streaming was used to indicate viability. If a cell was determined to be viable, then emission signals were collected following fluorescence light excitation at 495 nm and 440 nm to produce a pH dependent signal and a pH independent signal, respectively (Fig. 3). For each cell, a
The time-course of images was captured to show viability during the imaging window. In addition, for each cell imaged, several different focal planes were also captured.

Figure 4 shows the 495 nm (Fig. 4B, F) and 440 nm (Fig. 4C, G) emission signals for two microinjected cells. Besides these images are the ratio images produced by dividing the 495 pH-dependent signals by the 440 pH-independent signals (Fig. 4D, H). Figures 4D and 4H show non-homogenous signal between cortical, trans-vacuolar, and nuclear cytoplasm. Nuclear regions showed a greater ratio signal than cortical and trans-vacuolar regions. In general, regions containing more cytoplasm report greater ratios. These images suggest that the ratio signal from the BCECF-dextran dye is not exhibiting path-length independence.

As a possible explanation, the ratio signal may not behave as expected if the dye concentration is low in the cytosol. A low dye concentration reduces the value of the denominator of the ratio to values that approach background. When the value of the denominator in the ratio image is close to the background fluorescence, then the ratio values have poor pH resolution since small variations in the denominator easily influence the ratio value.

An inherent drawback to microinjection is that it is hard to control the volume of dye introduced into the plant cell. The differences in the ratio images observed between the two cells in figure 4 might be explained by large differences in the concentration of dye introduced into the cell.

Microinjection is a difficult process in BY-2 cells. Of 200 microinjection attempts, only three cells appeared to be viable 10 minutes after microinjection. Table 1 reports the average pH values for these cells, calibrated from a standard curve prepared in the Hepler laboratory (Rounds CM, unpublished data). In addition to displaying a non-homogenous signal throughout areas of the cytoplasm, the average pH values between cells do not agree.
Figure 4. Two tobacco BY-2 cells microinjected with BCECF-dextran and imaged using a 40x objective (1.20 NA) on an inverted fluorescence light microscope. (A,E) Differential interference contrast images. (B,F) Emission signal following excitation with 495 nm light with background subtracted. (C, G) Emission signal following excitation with 440 nm light with background subtracted. (D, H) Ratio image created by dividing 495 nm image by the 440 nm image. Ratio images are pseudo-colored using a 16-bit color lookup-table available as a standard lookup-table in ImageJ, running from a ratio of 0.6 (blue) to 2.3 (red).
Table 1. Mean cytosolic pH values and standard deviations for three cells microinjected with BCECF-dextran

<table>
<thead>
<tr>
<th>Sample Cell</th>
<th>Mean Sample pH</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell 1</td>
<td>6.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Cell 2</td>
<td>6.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Cell 3</td>
<td>6.2</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Together, the difficulty of the microinjection process, the observation of path-length dependence, and the unrepeatability of the results between sample cells suggested that microinjection of BCECF-dextran was not a robust method for reporting pH in BY-2 cells.

2.2 Biolistic particle bombardment of BCECF-dextran into BY-2 cells

Although microinjection of BCECF-dextran was not supported as a robust system for pH reporting in BY-2 cells, BCECF-dextran is a valuable pH reporter. Bothwell et al. (2006) showed that fluorescent dyes could be introduced into BY-2 cells by biolistic particle bombardment. Therefore, I loaded BCECF-dextran was loaded onto gold microcarriers according to Bothwell et al. (2006). These gold microcarriers were shot into BY-2 cells was not explored further.

Unfortunately, following bombardment with BCECF-dextran coated gold microcarriers BCECF fluorescence signal was undetectable. Since no signal was observed in BY-2 cells, bombardment of BCECF-dextran into BY-2 cells was not supported as a robust method for reporting pH.

2.3 Biolistic particle bombardment of pH-GFP into BY-2 cells

Biolistic particle bombardment is routinely used to introduce genes into plant cells. Expression of these genes is driven by a promoter to yield transiently transformed cells. This approach requires that the pH reporter is protein based. I received a gene encoding pH-GFP from the Gilroy Laboratory (University of Wisconsin). The protein, pH-GFP, is an engineered variant of eGFP with an amino-acid substitution at the 148th amino-acid position from histidine to aspartic acid. This amino-acid substitution renders the pH-GFP protein more sensitive to pH changes than eGFP. Furthermore, the switch also influences the fluorescence properties of the pH-GFP molecule. Similar to BCECF,
excitation of the pH-GFP with fluorescent light at 495 nm produces a signal that is pH dependent and excitation of pH-GFP with fluorescent light at 440 nm produces a signal that is pH independent (Figure 2, Elsliger et al., 1999; Fasano et al., 2001).

The plasmid, containing pH-GFP, received from the Gilroy Laboratory, was previously used for expression of pH-GFP in *Vicia faba*. With this plasmid, the expression of pH-GFP is driven by a 35S promoter. The plasmid was grown in *E. coli* and extracted by maxi-prep at high concentrations. DNA was ethanol precipitated onto gold microcarriers and these were shot into BY-2 cells using a pneumatic particle gun.

Transient transformation of BY-2 cells was observed within six hours following bombardment. Cells were routinely imaged 24 hours following bombardment, indicating that expression was not toxic to BY-2 cells within a 24-hour time window. The yield of transiently transformed cells was low (<1%). Figure 5 shows three cells transiently transformed with pH-GFP introduced by biolistic particle bombardment. Although the yield of transiently transformed cells was low, repeated bombardments yielded similar frequencies of transformants. Signals from dependent (Fig. 5B, F, J) and independent (Fig. 5C, G, K) emission channels were manipulated as for BCECF microinjections to produce a set of ratio images (Fig. 5D, H, L).

Ratio images produced from the bombardment of the gene encoding pH-GFP into interphase BY-2 cells showed relatively homogenous cytosolic ratio signals (Fig. 4D, H, L). A uniform ratio signal is expected from a ratiometric pH reporter if the pH of the cytosol is spatially uniform. When the average ratios from areas of cytosol corresponding to cortical, trans-vacuolar, and nuclear regions were reported, small differences were observed and these differences were not evidently biased by the thickness of the observed region (Fig. 6A, B, C). These results contrast with the path-length dependent ratio signal produced from microinjection of BCECF-dextran (Fig. 3) and suggest that pH-GFP is a robust reporter of intracellular pH.
Figure 5. Three tobacco BY-2 cells following biolistic bombardment with the gene encoding pH-GFP, imaged using a 40x objective (1.30 NA). (A, E, I) Differential interference contrast images. (B, F, J) Emission signal following excitation with 495 nm light with background subtracted. (C, G, K) Emission signal following excitation with 440 nm light with background subtracted. (D, H, L) Ratio images. Ratio images are pseudo-colored using a 16-bit color lookup-table available as a standard lookup-table in ImageJ, running from a ratio of 0.6 (blue) to 2.3 (red).
Table 2 shows the reported ratios for six interphase cells expressing pH-GFP following transient transformation mediated by particle bombardment. One drawback to the use of pH-GFP is that it is not calibrated as easily as BCECF-dextran. While BCECF-dextran can be calibrated in buffered solutions of pseudo-cytosol, pH-GFP must first be expressed and purified in vivo before being introduced into buffered solutions of pseudo-cytosol. Therefore, I report ratio averages only. Interestingly, the average ratios for cells collected on any given day had a low standard error while the average ratios of cells collected over a three month period had much greater variability. When the average of the sample means for a given day was plotted as a function of progressive imaging day (Fig. 6), the ratio averages seemed to vary with time. Since it is unlikely that properties of the pH-GFP or the BY-2 cells changed as a function of imaging date, these results implicate instrument conditions (for example, the output of the bulb) and should be taken as caution for ratiometric fluorescence measurements. Calibration of a reporter might only be applicable for a short time window.

Although bombardment of BY-2 cells with plasmid-encoded pH-GFP was shown to be a reproducible method of generating transiently transformed cells, several drawbacks led me to conclude that this was not a robust system for reporting cytosolic pH. First, yield of transiently transformed cells was low. Since one aim of my research is to use this system to investigate pH patterns throughout the cell cycle, an ideal pH reporter system should provide a yield of transformed cells of at least 20% for applications such as cell-cycle synchronization. Second, biolistic particle bombardment does have toxic effects on plant cells (Russell et al., 1992) and this could influence the pH physiology of the plant cell.

Optimistically, these experiments demonstrated that expression of pH-GFP in plant cells is a robust reporter for cytosolic pH. The cytosolic pH-GFP did not show any tell-tale path-length dependence characteristics as exhibited by microinjected BCECF-
dextran. I therefore adopted pH-GFP as a cytosolic pH reporter and investigated other methods for introducing the pH-GFP gene into plant cells to maximize the efficiency of transformation while minimizing the trauma imposed on the plant cell.

2.4 *Agrobacterium* mediated transient transformation of BY-2 cells with pH-GFP

Since pH-GFP was supported as a robust ratiometric indicator of intracellular pH, I explored *Agrobacterium* mediated transient transformation as another approach for introducing the pH-GFP gene into BY-2 cells. *Agrobacterium* mediated transformation is carried out by the gram-negative bacteria *A. tumefaciens*. Typically, *Agrobacterium* mediated transformation is used in-conjunction with antibiotic selective pressure to generate a stable transgenic line in plant cells. However, Buschmann et al. (2010) showed that *Agrobacterium* could be used to mediate transient transformation of plant cells in the absence of antibiotic selection with transformation efficiencies close to 20%. I adapted a protocol for agrobacteria mediated transient transformation based on the protocol described by Buschmann et al. (2010) and attempted to replicate their results.

Briefly, I obtained pGWB2, a gateway compatible binary vector, from the Nakagawa Laboratory (Nakagawa, 2002; Nakagawa et al., 2007). The gene encoding pH-GFP was directionally cloned into pGWB2 via the Gateway pENTR entry vector. When the gene encoding pH-GFP was cloned into the pGWB2, it was again placed under the control of a 35S promoter. The pGWB2 plasmid containing pH-GFP was transformed into three different strains of *A. tumefaciens*: EHA105, LBA4404, and GV3101.

Cells from BY-2 cultures and each of the *A. tumefaciens* strains were grown in liquid culture until log-phase growth was achieved. *A. tumefaciens* strains were subcultured approximately 5 hours prior to transformation in the presence of 250 µM acetylsyringone, a known elicitor of *Agrobacterium* conjugation, until OD$_{600}$ of 0.8 was achieved. BY-2 cells and *A. tumefaciens* cells were washed with Paul’s medium and co-
Figure 6. (A) Differential interference image of a cell bombarded with the gene encoding pH-GFP and (B) the corresponding 495 signal image. Dots indicate regions sampled to evaluate path-length dependence attributes of the pH-GFP dye: blue (cortical region), green (nuclear region), red (trans-vacuolar strand). (C) Ratio averages for various regions, for one cell. (D) Mean of ratio averages for populations of cells grouped by imaging day. Error bars indicate standard errors of the means.
Table 2. Mean cytosolic signal ratio values and standard errors of the means for populations of cells bombarded with the gene encoding pH-GFP on six imaging

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of Cells</th>
<th>Mean Ratio</th>
<th>SEM</th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.950</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.17</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1.29</td>
<td>0.06</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1.65</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1.67</td>
<td>0.11</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>1.99</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Average Over Days: 1.45
cultured in liquid Paul’s medium for five minutes. After five minutes, 20 µL droplets of cells were spotted onto plates of solid Paul’s medium, and then 250µM acetosyringone was added to each cell droplet. These plates were incubated for 48 hours at 26°C. After 48 hours, cells were examined for expression.

When the protocol described by Buschmann et al. (2010) was followed as closely as possible, transiently transformed cells were observed with a frequency less than that observed for bombardment (1 or 2 cells per plate, <<1%) (Fig. 7). Transformation was only observed when the strain EHA105 was.

*Agrobacterium* mediated transient transformation offers the potential to generate pH-GFP expressing transformants while minimizing stress placed on BY-2 cells. Since these characteristics are qualities that I identified as valuable in a robust pH reporter system, I decided to troubleshoot this protocol to increase the yield of transiently transformed cells.

### 2.5 Troubleshooting *Agrobacterium* mediated transient transformation of BY-2 cells

To troubleshoot the *Agrobacterium* mediated transient transformation protocol, I identified variables in the Bushmann et al. (2010) protocol which seemed likely to affect the frequency of observed transient transformation. Since EHA105 was the only strain that I observed to mediate transient transformation when the Buschmann et al. (2010) protocol was followed, and since this agreed with the results presented by Buschmann et al. (2010), I used the strain EHA105 for all further troubleshooting. First, I varied the concentration and application window of acetosyringone treatment. Second, I varied the time of liquid co-cultivation between BY-2 cells and *A. tumefaciens* cells. Third, I varied the solid-agar co-cultivation window. Fourth, I varied the concentration of BY-2 cells and *Agrobacterium* cells in the liquid co-cultivation step. Fifth, I varied the co-cultivation medium from Paul’s medium to standard BY-2 medium. Finally, I experimented with cell-wall wounding agents as elicitors of conjugation to supplement acetosyringone.
Figure 7. BY-2 cells transiently transformed with pH-GFP mediated by *Agrobacterium* and plant-cell conjugation. BY-2 cells were co-cultivated with *Agrobacterium* harboring pGWB2 containing pH-GFP after 48 hours. Cells were imaged using a 20x objective on an upright fluorescence light microscope.

Figure 8. BY-2 cells transiently transformed with pH-GFP mediated by polyethylene glycol treatment. Cells were imaged using a 20x objective on an upright fluorescence light microscope 24 hours following transformation.
Acetosyringone is a well described elicitor of conjugation between Agrobacterium and plant cells and is frequently used to increase the efficiency of transformation. It is possible that changes to the concentration or the application of acetosyringone could have a dramatic effect on the efficiency of transient transformation. I first varied the concentration of acetosyringone uniformly in the Agrobacterium log-phase subculture step and in the drops added to the solid medium. I created dilutions of acetosyringone in Paul’s medium at various concentrations between 100 µM and 5 mM. At concentrations above 500 µM, increased autofluorescence was observed 48 hours after the start of solid medium co-cultivation. Autofluorescence is likely an indication of cell stress. No transient transformation events were observed for treatments of acetosyringone above 250 µM. Interestingly, no reduction in the frequency of transformation was observed when the concentration of acetosyringone was lowered to 100 µM.

Next, I varied the timing of the acetosyringone application treatment while the concentration of acetosyringone was maintained at 250 µM. I first removed acetosyringone from the Agrobacterium log-phase subculture step. Next, I removed acetosyringone from the solid medium co-cultivation dropper step. When either of these treatments was carried out, I observed no transient transformation events, suggesting that these treatments, when used together, do modestly increase the frequency of transient transformation.

I varied the time period of the liquid co-cultivation step. Buschmann et al. (2010) suggested a 5 minute liquid co-cultivation step. In repeated trials, I observed cells for changes in the frequency of transient transformation following no liquid co-cultivation step (cells were suspended briefly in Paul’s medium and agitated to homogenize the distribution of cells), a 10 minute, a 20 minute, a 1 hour, and a 2 hour liquid co-cultivation step. For each of these treatments, no changes to the frequency of transient transformation were observed.
Buschmann et al. (2010) suggested a 48 hour solid medium co-cultivation period before cells should be observed for transient transformation. However, they also showed that solid medium co-cultivation increased the frequency of transient transformation over an extended liquid co-cultivation step. I hypothesized that the time of solid medium co-cultivation would also influence the frequency of observed transient transformants. I repeated the transient transformation protocol and observed cells for transgene expression after 24, 48, 36, 168, and 336 hours. For each of these trials, I observed no increased expression frequency. For solid medium co-cultivation times above 36 hours, I observed increased autofluorescence. This increased autofluorescence is likely secondary to the increased concentration of *Agrobacterium* on growth plates, as *Agrobacterium* quickly cover the surface of cell colonies and plates in the absence of antibiotic selection.

It is possible that the efficiency of transient transformation is sensitive to the proportion of BY-2 cells to *Agrobacterium* cells in the liquid co-cultivation step. I adjusted this ratio by varying the concentration of *Agrobacterium* in the co-cultivation step. I repeated the Buschmann et al. (2010) protocol in trials where the concentration of *Agrobacterium* in the liquid co-cultivation step was doubled and tripled. In neither case was an increase in the frequency of transient transformation observed. When the concentration of *Agrobacterium* was tripled, increased autofluorescence was noticed in cells, possibly indicating that BY-2 cells were stressed by the high *Agrobacterium*.

Buschmann et al. (2010) found that the use of Paul’s medium for all BY-2 and *Agrobacterium* co-cultivation steps was superior to the use of BY-2 medium for these steps. Paul’s medium is the foundation of BY-2 media and contains Murashige and Skoog medium and sucrose (pH 5.7). Regular BY-2 growth medium also contains KH$_2$PO$_4$, thiamine, inositol, and 2,4-D. In case the use of BY-2 medium was beneficial for the transient expression of pH-GFP, I substituted BY-2 medium for Paul’s medium in both the liquid and solid co-cultivation steps. When I carried out these treatments, I
observed no cases of transient transformation, suggesting that Paul’s medium has some benefit to the frequency of transient transformation.

Finally, I explored the use of additional elicitors of conjugation to supplement the use of acetosyringone. Cell-wall wounding has been described as an elicitor of conjugation between *Agrobacterium* and plant-cells. To cause cell-wall wounding, I subjected log-phase BY-2 cells to 10 and 20 minute treatments with low concentrations of pectinase and cellulase (0.02%). I washed BY-2 cells with Paul’s medium before beginning the liquid co-cultivation step with *Agrobacterium*. In the case of both treatments, I did not observe an increase in the frequency of transient transformation. I did observe increased autofluorescence for cells pre-treated with pectinase and cellulase indicating that these treatments did cause cell-stress.

I was unable to reproduce the results described by Buschmann et al. (2011). To further troubleshoot this procedure, I carried out colony PCR analysis on each of the *Agrobacterium* strains. For each strain, I amplified pH-GFP from DNA within the cells indicating that these cells were successfully transformed with pGWB2 carrying pH-GFP.

### 2.6 PEG mediated transient transformation of BY-2 cells with pH-GFP

There are three canonical approaches to plant-cell transformation. These are: (1) biolistic bombardment, (2) *Agrobacterium* mediated transformation, and (3) direct transformation, typically aided by transient membrane disruption, for example with polyethylene glycol. To explore all possible approaches to transient transformation of BY-2 cells with the gene encoding pH-GFP, I investigated the use of polyethylene glycol. Polyethylene glycol mediated transformation, in plant-cells, requires a pre-treatment with cell-wall digesting enzymes to remove the cell-wall. After digestion, the plant-cells are maintained in a solution with ~0.5 M osmoticum to compensate for the removal of the cell wall. Polyethylene glycol treatment helps facilitate the movement of DNA into plant
cells. I used a protocol designed by Dr. Wenjuan Fang, a post-doctoral researcher working in the Baskin Laboratory (unpublished), to transform BY-2 cells with pH-GFP.

Following transformation, a low-frequency of transformation events was observed (Fig. 8). These transformation events were hard to distinguish from autofluorescence and needed long exposure times, around 5 seconds. To help characterize the expected response of cytosolic eGFP under the control of a 35S promoter in BY-2 cells following polyethylene glycol-mediated transformation, I used a plasmid containing eGFP under the control of a 35S promoter. I transformed both pH-GFP and EGFP into BY-2 cells using polyethylene glycol-mediated transformation in parallel treatments. I observed a higher frequency of transformation events and greatly increased diversity of fluorescence activity in the eGFP control trial. This suggests that polyethylene glycol mediated transformation is a robust method for introducing cytosolic GFP into plant-cells.

To troubleshoot the absence of pH-GFP signal in polyethylene glycol mediated transformation trials, I propose two explanations. First, it is possible the mutagenesis at the 148\textsuperscript{th} amino-acid position that characterizes pH-GFP over EGFP rendered the pH-GFP signal greatly diminished in comparison to EGFP. However, this possibility does not agree with the use of pH-GFP in the Gilroy Lab (Monshausen et al., 2009) who observed a strong cytosolic pH-GFP signal in \textit{A. thaliana} seedlings. Second, I propose that some property of the plasmid, such as the promoter region, may be diminishing the expression of pH-GFP in the BY-2 cytosol. If this is the case, cloning of the pH-GFP gene into a similarly designed expression vector that is known to drive ectopic protein expression in BY-2 cells should resolve the issue.
CHAPTER 3

METHODS

3.1 BY-2 cell culture

The *Nicotiana tabacum* cell line, BY-2, was grown in standard BY-2 medium, comprising the following: 4.3 g/L Maurashige and Skoog basal salts (Sigma, M5524-10L), 30 g/L sucrose, 0.2 g/L KH$_2$PO$_4$, 0.1 g/L inositol, 1 mg/L thiamine, and 0.2 mg/L 2,4-D. The pH was adjusted by the addition of KOH to 5.7. The BY-2 medium solution was autoclaved.

BY-2 cells were grown continuously in 300 mL glass Erlenmeyer flasks containing 80 mL of sterile BY-2 medium. Incubator conditions were maintained at 26.0°C and culture flasks were continually agitated by a laterally revolving surface at 120 revolutions per minute.

Cells were sub-cultured once every seven days. Using aseptic technique, 1 mL of cells were removed from the confluent culture flask and placed into 80 mL of fresh BY-2 medium. The new culture flask was placed back into the incubation chamber with conditions as previously described.

3.2 Preparation of BY-2 cells for microinjection

An aqueous solution of 1% poly-lysine (high molecular weight; Sigma, P1399) was created. One day before microinjection, 5 mL of confluent BY-2 cells were aseptically removed from a confluent culture flask and placed into 45 mL of fresh BY-2 medium. This new flask was returned to the incubation chamber.

In preparation for microinjection, glass coverslips were affixed with nail polish over holes in glass plates to create optically thin glass wells. These wells were washed thoroughly with distilled and deionized water. The glass wells were treated in a plasma cleaner for 30 seconds. After plasma treatment, 2 µL of 1% poly-lysine solution were
evenly spread over the surface of the coverslip on the interior of the glass well. This surface was allowed to dry and protected from dust by plastic wrap.

Directly before microinjection, 0.5 mL of previously sub-cultured cells were mixed with 5 mL of 3% sucrose solution and forcefully expelled over the surface of the coverslip. Cells were repeatedly expelled across the surface of the coverslip until the desired density of cells was attained. 1 mL of fresh BY-2 medium was used to bathe cells in the well after they were fixed to the coverslip surface.

3.3 Microinjection of BCECF-dextran into BY-2 cells

BCECF conjugated to a 70 kDa dextran molecule was obtained from Invitrogen (Lot 49360A) and diluted to a working concentration of 0.1 mg/mL in 10 µL aliquots.

Directly before microinjection, borosilicate glass capillary tubes (World Precision Instruments, Item # 1B100F-4) were pulled using a vertical pipette puller (KOPF, Model 700 D) with pull times between 15 and 22 seconds to create micro-needles. These micro-needles were back-filled with 1µL of BCECF-dextran solution at a concentration of 0.1 mg/mL using a 10 µL ultra-pipette and flexible plastic tips with beveled ends. After the dye was backfilled into the micro-needle, the needle was further backfilled with water to create a continuous fluid path from the tip of the needle to the opposite end. The needle was connected to a hydraulic hand-operated pressure pump and to a boom arm to hold the needle over the stage of an inverted fluorescence microscope (Nikon Eclipse, TE300). The boom arm was manipulated by a three-dimensional micro-manipulator (Narishige Scientific Instruments, Model MO-103). The needle was positioned right at the edge of a BY-2 cell, slowly introduced into the cell, and BCECF was pressure injected into the cytosol using the hand-pump.

Following microinjection, the needle was carefully removed from the BY-2 cell. The cell was observed for vigorous cytoplasmic streaming 10 minutes after the removal of the needle. If the cell was still streaming after 10 minutes, then the cell was imaged.
3.4 Biolistic particle bombardment of BY-2 cells

For biolistic particle bombardment of BY-2 cells, 1 µm gold microcarrier particles (Bio-Rad, Catalog 1652263), coated with DNA or dye, were placed onto microcarrier disks in the pressure path of a biolistic particle gun (Bio-Rad, Model PDS-1000). Above the coated microcarriers disks, a 1100 psi rupture disk was used to control accelerative pressure generated by the gun. BY-2 cells were thickly plated onto 1% agar + BY-2 media in a 3 centimeter diameter area directly below the firing path of the gun. The biolistic particle gun was fired twice in rapid succession according to the operating manual. Following the final discharge, the BY-2 cells were immediately removed from the pressure chamber and spread over the entire agar surface of a Petri dish, supplemented with the addition of 70 µL of fresh BY-2 medium.

3.5 Coating of gold microcarriers with BCECF-dextran

For experiments where BCECF-dextran was bombarded into BY-2 cells on gold microcarriers, I adapted a protocol described by Bothwell et al. (2006).

To begin, a solution of 50 mg/mL of gold particles was vortexed vigorously for one minute then immediately bath-sonicated for 10 seconds. The 50 mg/mL stock solution was divided into 25 µL aliquots. One of these aliquots was centrifuged at 4000 rpm for 10 seconds. The supernatant was removed and 50 µL of 8 mM BCECF-dextran was added to the gold particles. The tube containing the gold particles and the BCECF-dextran was vortexed for 1 minute and bath-sonicated for 10 seconds. The tube was then allowed to incubate in the dark at room temperature for 15 minutes. The mixture was again vortexed for 1 minute. The mixture was centrifuged for 10 seconds at 4000 rpm and the supernatant was decanted. The pellet was washed with 100 µL of 100% ethanol and centrifuged for 10 seconds at 4000 rpm. The supernatant was removed and 40 µL of ethanol was added. The pellet was vortexed for 1 minute and then bath-sonicated for 10
seconds. The mixture was then further aliquoted into 10 µL installments for use on the same day. Microcarrier discs were coated in a low light environment and allowed to dry.

3.6 Coating of gold microcarriers with plasmid DNA

For experiments where plasmid DNA was bombarded into BY-2 cells on gold microcarriers, I adapted a protocol described in the Bio-Rad PDS-1000 operations manual. A stock mixture of 1 µm gold microcarrier particles (as above) at 30 mg/mL in 50% glycerol was created. This mixture was bath-sonicated for 1 minute and then vigorously vortexed for 1 minute. Immediately after vortexing, 30 µL of the mixture was transferred to a 1.5 mL microfuge tube. The tube was vigorously vortexed while 5 µL of DNA (1 µg/µL), 50 µL of 2.5 M CaCl₂ and 20 µL of 0.1 M spermidine were added to the mixture. The mixture was continually vortexed for an additional 3 minutes. The mixture was bath-sonicated for 10 seconds. The mixture was centrifuged for 10 seconds at maximum speed. The supernatant was discarded and replaced with 140 µL of 70% ethanol. The mixture was vortexed for 1 minute and then bath-sonicated for 10 seconds. The mixture was centrifuged for 10 seconds at maximum speed. The supernatant was discarded and replaced with 140 µL of 100% ethanol. The mixture was vortexed for 1 minute and then bath-sonicated for 10 seconds. The mixture was centrifuged for 10 seconds at maximum speed. The supernatant was removed and replaced with 60 µL of 100% ethanol. The pellet was resuspended by vortexing the tube for 3 minutes and then bath-sonicating the tube for 10 seconds. Immediately after the last bath-sonication step, two 30 µL volumes of the mixture were removed and placed directly on micro-carrier discs. The mixture was spread out evenly along the surface of the disc and allowed to dry before bombardment.
3.7 *Agrobacterium* mediated transient transformation of BY-2 cells

This protocol for *Agrobacterium* mediated transient transformation of BY-2 cells was developed from a protocol described by Buschmann et al. (2010).

Cells from a confluent BY-2 culture flask containing 80 mL of BY-2 medium were subcultured by aseptically transferring 5 mL of cells into a culture flask containing 45 mL of fresh BY-2 medium. This new culture flask was returned to the incubation chamber for 48 hours with conditions as described previously.

*Agrobacterium* cells were inoculated from freezer stocks approximately 24 hours before the start of the transient transformation protocol into LB medium with antibiotics. *Agrobacterium* cells were cultured at 24°C in a shaker at approximately 200 revolutions per minute. After 30 hours, 1 mL of confluent *Agrobacterium* culture was transferred to 4 mL of fresh LB medium containing antibiotics and, in some experiments, 250 µM acetosyringone. After 4 hours and repeatedly at half hour intervals afterwards, culture OD$_{600}$ was checked until culture confluence was observed in the range of OD$_{600}$ 0.5 to 0.8.

After 48 hours, the BY-2 culture flask containing 5 mL of inoculum in 45 mL of LB medium was removed from the incubator. Using aseptic technique, 50 mL of culture was washed twice using sterile Paul’s medium (BY-2 medium without inositol, thiamine, 2,4-D, or KH$_2$PO$_4$). The cell mixture was then concentrated to 10 mL.

In parallel procedures, the culture tube containing *Agrobacterium* was pelleted into a 1.5 mL Eppendorf tube and resuspended in 30 µL of Paul’s medium. To this tube, 1 mL of concentrated BY-2 cells were added. The tube containing BY-2 cells and *Agrobacterium* cells was gently agitated for 5 minutes. The BY-2 and *Agrobacterium* mixture was spotted onto plates containing Paul’s medium + 1% agar with no antibiotics in approximately 30 µL volumes using cut-end pipette tips. Acetosyringone solution at a concentration of 250 µM was pipetted onto the cell spots. The plate was incubated for 48
hours before cells were checked for transient expression using an upright fluorescence light microscope (Zeiss Axioplan)

3.8 Polyethylene glycol mediated transformation of BY-2 cells

For all protoplasting work, I used an unpublished protocol developed by Dr. Wenjuan Fang, a post-doctoral researcher working in the Baskin Laboratory. Several days before transformation, 1 mL of confluent BY-2 cells was aseptically transferred to a flask containing 45 mL of fresh BY-2 media. This new flask was returned to the incubator.

On the day of transformation, 3 mL of cells from the previously inoculated flask were mixed with 10 mL of enzyme digestion solution (by mass: 2% cellulase, 0.2% pectinase, 8.2% sorbitol, pH 5.5, filter sterilized) and allowed to incubate for 2 hours at 27°C. Following cell-wall digestion, protoplasts were strained and washed three times using wash solution (0.45 M sorbitol, 2 mM CaCl$_2$). The concentration of cells was counted using a hemocytometer and adjusted with MMG solution (0.45 M sorbitol, 15 mM MgCl$_2$, 4 mM MES, pH 5.7).

For transformation, 15 to 20 ng of DNA was mixed with 300 µL of cells and 300 µL of polyethylene glycol solution. This mixture was gently agitated and allowed to incubate at room temperature for 30 minutes. Next, 5 mL of washing solution was added. The mixture was allowed to incubate for an additional 30 minutes. The mixture was centrifuged (7 minutes at 250g) and the supernatant decanted. Protoplasts were resuspended in 3 mL of post-transformation culture medium (72.8 g/L mannitol, 4.3 g/L Maurashige and Skoog basal salts, 30 g/L sucrose, 0.2 mg/L 2,4-D, pH 5.7). This mixture was poured into plastic petri dishes and allowed to incubate for 24 hours. Protoplasts were viewed using an upright fluorescence light microscope (Zeiss Axioplan).
3.9 Molecular manipulation of the pH-GFP gene

In preparation for bombardment and polyethylene glycol mediated transformation, pH-GFP was obtained at a concentration of 1.5 \( \mu g/\mu L \) by a maxi-prep, following the protocol in Sambrook et al. (1989).

In preparation for transient transformation of tobacco BY-2 cells mediated by \textit{Agrobacterium}, the gene encoding pH-GFP was directionally cloned into the pENTR entry vector according to the pENTR/dTOPO cloning manual (Invitrogen). An LR clonase reaction was carried out according to the LR Clonse II reaction manual (Invitrogen) to clone pH-GFP from pENTR into pGWB2 (Nakagawa, 2002; Nakagawa et al., 2007). The destination vector pGWB2 harboring pH-GFP was transformed into the \textit{Agrobacterium} strains EHA105, GV3101, and LBA4404, and the presence of pH-GFP was confirmed in these strains by colony polymerase chain reaction and sequenced.

In preparation for \textit{in vitro} calibration of pH-GFP, the gene encoding pH-GFP was amplified and cloned into the pGEM vector according to the pGEM/pGEM T-Easy system protocol (Promega, 2010).

All mini-preps were carried out using the Wizard Plus SV minipreps DNA Purification System (Promega, Cat. A1330). All gel extractions and purifications were carried out using the Wizard SV Gel and PCR Clean-Up System (Promega, Cat. 301884). All gels were created as 1\% agarose gels in TBE buffer.
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