Letter

Genetically encoded calcium indicators for fluorescence imaging in the moss Physcomitrella: GCaMP3 provides a bright new look

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The moss Physcomitrella patens is emerging as a model organism due to the availability of established protocols for precise genome engineering by homologous recombination and a high-quality genome sequence (Rensing et al., 2008). Physcomitrella is an attractive system for cell biology and live cell imaging and has been used to investigate fundamental cellular processes such as tip growth, vesicle trafficking and cytoskeletal dynamics (e.g., Furt et al., 2012). In each of these processes, calcium (Ca²⁺) acts as an important signal to relay developmental and environmental cues. Classical techniques have been used to monitor Ca²⁺ dynamics in this early land plant model. Using the luminescent Ca²⁺ reporter aequorin, osmotic shock or mechanical perturbations were shown to elicit transient Ca²⁺ signals in Physcomitrella (Haley et al., 1995; Russell et al., 1996), but this approach provided limited spatial information about these responses. Alternatively, Physcomitrella cells have been biolistically loaded with synthetic fluorescent Ca²⁺ indicator dyes to visualize saline-elicted Ca²⁺ signals (Qudeimat et al., 2008); however, this approach damages the cells used for Ca²⁺ imaging and is poorly suited for long-timescale experiments because of dye sequestration and dilution. To extend the Ca²⁺-imaging toolkit for Physcomitrella, we engineered lines transformed with genetically encoded calcium indicators (GECIs) and assessed their performance using conditions known to trigger transient Ca²⁺ changes.

Two GECIs, Yellow Cameleon 3.6 (YC3.6) (Nagai et al., 2004) and GCaMP3 (Tian et al., 2009), were selected for in vivo evaluation in Physcomitrella. At their core, each sensor contains an engineered Ca²⁺-binding calmodulin (CaM) variant and CaM-binding peptide fused to one or two fluorophores that optically report Ca²⁺-dependent conformational changes. The YC3.6 sensor contains a cyan fluorophore (enhanced cyan fluorescent protein, eCFP) and a yellow fluorophore (cpVENUS), which can accept energy from eCFP through Förster Energy Resonance Transfer (FRET); FRET magnitude is positively correlated with Ca²⁺ concentration. The more recently developed GCaMP3 sensor contains a single circularly permuted green fluorescent protein (cpGFP); and fluorescence intensity (FI), which is positively correlated with Ca²⁺ concentration, is directly used as the signal readout. FRET-based GECIs such as YC3.6 have been widely used in higher plant systems, however, GCaMP3 and related GECIs reportedly offer superior performance in vitro and in animal systems (reviewed by Rodriguez et al., 2017). In Arabidopsis thaliana, R-GECO1, which was engineered from GCaMP3 by replacing cpGFP with the red fluorophore cpmApple (Zhao et al., 2011), has been reported to offer greater response magnitude compared to YC3.6 (Keinath et al., 2015). To our knowledge, there are no publications so far that report similar comparisons using GCaMP3 or related green GECIs in any plant or that describe use of fluorescent GECIs in a bryophyte.

To engineer Physcomitrella lines expressing fluorescent GECIs, protenomal cells were cultured on cellophane-overlaid agar growth media and bombarded with DNA-coated gold particles. Expression of YC3.6 or GCaMP3 was driven by a Zea mays Ubiquitin1 promoter with its 5′ leader intron. Because GCaMP3 is an intensiometric sensor, we co-transformed GCaMP3 lines with Porites porites red fluorescent protein (RFP) driven by a Panicum virgatum Ubiquitin1 promoter to serve as an internal reference and control. Fluorescent signal could be detected 48 h after bombardment (Figure S1), and transformed cells were selected by antibiotic resistance. Colonies that grew under the first round of selection were embedded in antibiotic-supplemented media and maintained under sterile conditions in growth chambers (Appendix S1). This method has allowed us to rapidly generate populations of stably transformed Physcomitrella lines that have been maintained over 3 years (to date) by periodic subculture. Transgenic lines expressing YC3.6 were prepared for Ca²⁺ imaging by growing small colonies on cellophane-overlaid plates and transferring them to coverslips with media reservoirs (Appendix S1). Spinning disc confocal microscopy was used to collect emissions from the donor fluorophore under direct excitation (DxDm), the acceptor fluorophore...
under donor excitation (DxAm) and the acceptor fluorophore under direct excitation (AxAm). Salinity stress was applied by stepwise increases in NaCl concentration (Figure 1a, Movies S1). Each addition of NaCl provoked a transient (2–3 min) increase of approximately 3%–7% in DxAm FI across the field of view and a corresponding decrease of similar magnitude in DxDm FI (Figure 1b). At approximately 350 mM NaCl, cells plasmolysed, and FIs decreased by approximately 40% in each channel. The control treatment using media lacking NaCl did not elicit FI changes that could be discriminated from background noise; thus, the YC3.6 sensor is suitable for monitoring salinity-elicited Ca\textsuperscript{2+} signalling events in Physcomitrella.
When sensor readout is plotted as the DxAm/DxMm ratio, there is a clear decline in the DxAx/DxMm ratio: the initial resting-state value of 1.25 gradually falls to 1.10—roughly a 14% decrease—just before the final treatment leading to plasmolysis. We also observed a ~14% decrease in the AxTx channel over the ~37 min experiment; therefore, the decrease in ratio is likely due to acceptor photobleaching. Further experimentation may allow for improved postacquisition data processing; however, we decided to test whether GCaMP3 may offer superior performance without requiring substantial postacquisition analysis.

Transgenic lines expressing GCaMP3 and the internal reference RFP were prepped for salt shock experiments using the same procedure as YC3.6 lines. After a mock control, which was performed by addition of media lacking NaCl and did not elicit signal intensity changes, cells were treated with 100 mM NaCl. The treatment elicited a pronounced spike in GCaMP3 FI, whereas RFP signal intensity remained steady throughout the experiment (Figure 1c, Movie S2). At its peak, which occurred approximately 2 min after the initial salt shock, the GCaMP3 FI dropped ~45% across the field of view. Signal returned to baseline intensity within 5 min of treatment without removal or dilution of NaCl (Figure 1d). We did not encounter noticeable photobleaching of GCaMP3 or RFP over the course of the ~50-min experiment. Our data collectively suggest that GCaMP3 is a simple, reliable GECI to use in Physcomitrella that offers advantages over YC3.6 in terms of photostability and response magnitude and requires less postacquisition data processing.

The striking performance of GCaMP3 in Physcomitrella protoneural cells during salt shock assays prompted us to monitor mechanically evoked Ca\(^{2+}\) signals in this system. Mechanical responses in cells expressing GCaMP3 were assayed by stimulation with a round-tipped glass microprobe mounted on a motorized micromanipulator. Transient elevations in GCaMP3 FI were elicited by touching cells with the microprobe and were locally confined near the stimulated region (Figure 1e,f; Movie S3). Similar FI increases were not observed in the RFP channel. Mere movement of the probe next to cells, without making contact, did not elicit this response, implying that mechanical stimulation was responsible for observed Ca\(^{2+}\) transients. This simple demonstration elegantly reveals subcellular Ca\(^{2+}\) dynamics associated with mechanical stimuli and suggests that GCaMP biosensors may be useful tools to investigate plant mechanoreception.

In this study, we engineered transgenic Physcomitrella lines expressing fluorescent biosensors YC3.6 or GCaMP3 and validated their utility for Ca\(^{2+}\) imaging, while highlighting the advantages of GCaMP3. Combined with currently available molecular genetic tools for Physcomitrella, the simple and rapid method for biosensor deployment presented here provides a promising approach for phenotypic interrogation of mutant lines. Going forward, this method can be used to engineer moss lines that express other GECIs, such as the more newly developed GCaMP6 series, or biosensors for other molecules, such as metabolites or hormones.

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Conflict of interest
The authors declare no conflict of interest.

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Supporting information
Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Fluorescent signal from Physcomitrella cells successfully transformed with RED FLUORESCENT PROTEIN (RFP) using particle bombardment.

Appendix S1 Materials & Methods.

Movie S1 Imaging saline-elicited Ca\(^{2+}\) signals in Physcomitrella using the FRET-based sensor YC3.6.

Movie S2 Imaging saline-elicited Ca\(^{2+}\) signals in Physcomitrella transforming using GCaMP3.

Movie S3 Imaging touch-elicited Ca\(^{2+}\) signals in Physcomitrella transforming using GCaMP3.

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