Topic Introduction

Ca²⁺ Imaging in Plants Using Genetically Encoded Yellow Cameleon Ca²⁺ Indicators

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Temporally and spatially defined changes in cellular calcium (Ca²⁺) concentration represent stimulus-specific signals and regulate a myriad of biological processes. The development of ratiometric Ca²⁺ reporter proteins like Yellow Cameleons (YCs) has greatly advanced our ability to analyze Ca²⁺ dynamics in vivo with unprecedented spatial and temporal resolution. In plants, the application of these Ca²⁺ reporter proteins has been pioneered for the investigation of Ca²⁺ dynamics in guard cells, and recently their use has been extended to other single-cell models like growing pollen tubes and root hairs. However, in plants, the use of YC reporter proteins has largely remained restricted to the investigation of cytoplasmic alterations of Ca²⁺ concentrations. Here, we provide an introduction to current methods for imaging Ca²⁺ dynamics with increasing sophistication.

INTRODUCTION

Calcium (Ca²⁺) represents one of the most versatile second messengers in all eukaryotic organisms. In plants, Ca²⁺ is involved in nearly all aspects of development and participates in many regulatory processes (Dodd et al. 2010; Kudla et al. 2010). Ca²⁺ signals are perceived as transient increases in cytosolic free Ca²⁺ concentration ([Ca²⁺]_c) arising through fluxes from external sources and internal subcellular compartments (Hetherington and Brownlee 2004; Dodd et al. 2010). Specificity in Ca²⁺ signaling is achieved (at least in part) by stimulus-specific, spatially and temporally distinct changes in cellular Ca²⁺ concentrations, designated as “Ca²⁺ signatures” (Webb et al. 1996). Specific Ca²⁺ signatures that are formed in response to different stimuli—including drought, salt, or osmotic stresses, temperature, light, and plant hormones—occur as a result of the tightly regulated activities of channels and transporters in different membranes (Dodd et al. 2010; Kudla et al. 2010).

The visualization and analysis of intracellular Ca²⁺ dynamics in living plants was revolutionized through the introduction of genetically encoded Ca²⁺ indicators (GECIs): aequorin, GFP-based Ca²⁺ probes, and, very recently, BRET-based indicators (Saito et al. 2012), detected either by photon emission measurements or fluorescence microscopy. These developments were facilitated by the relative ease of obtaining stably transformed transgenic plants, especially tobacco and *Arabidopsis*, and by the simplicity of storing and propagating the transgenic lines as seeds. (For further details on

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plant transformation and propagation, refer to *Arabidopsis Protocols* [Salinas and Sanchez-Serrano 2006]). The first tools developed for in vivo intracellular analyses of Ca²⁺ dynamics in plants were based on aequorin targeted to the cytoplasm (Knight et al. 1991), nucleus (van Der Luit et al. 1999), chloroplasts (Johnson et al. 1995), tonoplast (Knight et al. 1996), and mitochondria (Logan and Knight 2003). Recently, aequorin has been targeted to the stroma as well as the inner and outer chloroplast envelope membranes (Mehlmer et al. 2012). These experiments produced very reliable data, but merely reflected the response of a population of cells or plants; hence, intercellular heterogeneity could not be investigated. The limited resolution and rather low emission intensity of aequorin measurements underscored the need for an advanced Ca²⁺ detection system with the combined ability to record Ca²⁺ transients at high resolution and express the Ca²⁺ indicator in the desired tissues or intracellular locations.

YELLOW CAMELEON Ca²⁺ INDICATORS

In recent years, the development and application of ratiometric Ca²⁺ reporter proteins (YCs or Cameleons) has greatly advanced the spatial and temporal resolution of Ca²⁺ signaling studies. Consequently, these Ca²⁺ sensors have been increasingly used among the GECIs that are available for such studies in plants. YCs are fluorescence resonance energy transfer (FRET)-based indicator proteins (Fig. 1). They harbor two GFP variants, CFP and YFP (or circularly permuted variants of YFP) linked together by the Ca²⁺-binding protein calmodulin and the calmodulin-binding peptide M13. Binding of Ca²⁺ to the calmodulin of YC leads to a conformational change in the indicator,

FIGURE 1. The functional principle of the genetically encoded Ca²⁺ indicator Yellow Cameleon (YC). (A) Functional components of the Cameleon probe: the two fluorophores CFP and YFP, the calcium binding element CaM, and the M13 connector. (B) Demonstration of FRET between CFP and YFP as the fluorophores move into close proximity due to a conformational change after the binding of Ca²⁺ to CaM. (C) Absorption and emission spectra of CFP and YFP, showing spectral overlap between CFP emission and YFP excitation. This spectral overlap allows excitation of YFP by CFP emission. (D) Read-out of a typical Cameleon measurement showing the emission intensity of CFP (donor) and YFP (acceptor) after CFP excitation. (E) FRET efficiency measured as the ratio between acceptor (YFP) emission and donor (CFP) emission.

bringing CFP and YFP into close proximity and allowing enhanced FRET between CFP and YFP. The efficiency of FRET allows quantitative measurements of Ca^{2+} dynamics to be made by recording the ratio shifts along a time-course.

Allen et al. (1999) reported the first successful application of YC in plants, using the YC2.1 version to monitor Ca^{2+} dynamics in guard cells. During the last few years, new YC variants with improved fluorescence, larger changes in FRET on Ca^{2+} binding, and broader ranges of Ca^{2+} affinities have been developed (Palmer and Tsien 2006; Palmer et al. 2011). With these YC variants, it has now become possible to monitor Ca^{2+} in different organs and tissues and subcellular compartments. Plants expressing the improved YC3.6 version of Cameleon (Nagai et al. 2004) were used to record cell type-specific Ca^{2+} oscillations evoked by extracellular nucleoside-triphosphates (Tanaka et al. 2010) and toxic metals (Rincón-Zachary et al. 2010), and have been used to study events in growing root hair tips (Monshausen et al. 2008), mechanically stimulated roots (Monshausen et al. 2009), and pollen tubes (Iwano et al. 2009). Nuclear- and plasma membrane-targeted YC3.6 allow the visualization of dynamic changes in Ca^{2+} concentration within the nucleus and in the subplasma membrane compartment (Sieberer et al. 2009; Krebs et al. 2012).

A further advantage of YC is that it can be easily adapted for different Ca^{2+} binding affinities. This feature enabled the recent investigation of Ca^{2+} dynamics in the endoplasmic reticulum (which contains high concentrations of Ca^{2+}) using version YC4.6 (Iwano et al. 2009). Moreover, in the D family of Cameleon indicators (Palmer et al. 2006), the basic structure of the probe remains unchanged, but both calmodulin (CaM) and the calmodulin-binding peptide have been mutated to abolish or strongly reduce the interference of the reporter protein with endogenous calmodulin. The first use of the D3cpv version of Cameleon in plants enabled the monitoring of Ca^{2+} dynamics in plant peroxisomes (Costa et al. 2010), where CaM is indeed present (Chigri et al. 2012). The most recent, successful implementation of mitochondria-targeted YC3.6 enhanced the understanding of Ca^{2+} dynamics in the mitochondria of both guard and root cells in plants (Loro et al. 2012). Additionally, concurrent expression of differentially targeted YC3.6 reporters in the same cell facilitated simultaneous recordings of mitochondrial and nuclear Ca^{2+} dynamics in *Arabidopsis* (Loro et al. 2012).

ANALYZING Ca^{2+} DYNAMICS IN PLANT CELLS

We have provided a set of detailed protocols describing current, well-established techniques and methodologies for using YC indicators. This compilation should provide the interested user with a broad tool kit to study Ca^{2+} dynamics in various contexts. We begin with a basic protocol for analyses of cytoplasmic Ca^{2+} dynamics in guard cells in **Live Cell Imaging of Cytoplasmic Ca^{2+} Dynamics in *Arabidopsis* Guard Cells** (Behera and Kudla 2013a). In **High-Resolution Imaging of Cytoplasmic Ca^{2+} Dynamics in *Arabidopsis* Roots** (Behera and Kudla 2013b), we extend this repertoire to include analyses of cytoplasmic Ca^{2+} in complex tissues. A third protocol, **Live Cell Imaging of Cytoplasmic and Nuclear Ca^{2+} Dynamics in *Arabidopsis* Roots** (Krebs and Schumacher 2013), advances the use of spatial resolution to achieve the combined imaging of cytoplasmic and nuclear Ca^{2+} . The final protocol, **Imaging of Mitochondrial and Nuclear Ca^{2+} Dynamics in *Arabidopsis* Roots** (Loro and Costa 2013), further describes development of this technique for simultaneous recording of mitochondrial and nuclear Ca^{2+} dynamics.

These readily implementable methods will allow users to pursue Ca^{2+} imaging and analyses in guard and root cells. Our descriptions are focused on the most versatile YC3.6 version of Cameleon probe and its analysis using both wide-field fluorescence and laser scanning confocal microscopy. We have paid particular attention to describing the preparation and manipulation of transgenic plants, and provided in-depth instructions for performing imaging analyses in cells from different organs and tissues and in different subcellular compartments. Thus, these protocols will enable the reader to successfully perform Cameleon-based Ca^{2+} imaging recordings in different plant cell types, using various transgenic plants available to the broader scientific community (Yang et al. 2008; Krebs et al.

2012; Loro et al. 2012). Moreover, we hope that this compendium of methods for the study of Ca²⁺ dynamics will aid the development of additional, novel approaches that further advance our understanding of this important phenomenon in plant biology.

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REFERENCES

- Allen GJ, Kwak JM, Chu SP, Llopis J, Tsien RY, Harper JF, Schroeder JI. 1999. Cameleon calcium indicator reports cytoplasmic calcium dynamics in *Arabidopsis* guard cells. *Plant J* 19: 735–747.
- Behera S, Kudla J. 2013a. Live cell imaging of cytoplasmic Ca²⁺ dynamics in *Arabidopsis* guard cells. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot072983.
- Behera S, Kudla J. 2013b. High-resolution imaging of cytoplasmic Ca²⁺ dynamics in *Arabidopsis* roots. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot073023.
- Chigri F, Flösdorff S, Pilz S, Kölle E, Dolze E, Gietl C, Vothknecht UC. 2012. The *Arabidopsis* calmodulin-like proteins AtCML30 and AtCML3 are targeted to mitochondria and peroxisomes, respectively. *Plant Mol Biol* 78: 211–222.
- Costa A, Drago I, Behera S, Zottini M, Pizzo P, Schroeder JI, Pozzan T, Lo Schiavo F. 2010. H₂O₂ in plant peroxisomes: An in vivo analysis uncovers a Ca²⁺ dependent scavenging system. *Plant J* 62: 760–772.
- Dodd AN, Kudla J, Sanders D. 2010. The language of calcium signaling. *Annu Rev Plant Biol* 61: 593–620.
- Hetherington A, Brownlee C. 2004. The generation of Ca²⁺ signals in plants. *Annu Rev Plant Biol* 55: 401–427.
- Iwano M, Entani T, Shiba H, Kakita M, Nagai T, Mizuno H, Miyawaki A, Shoji T, Kubo K, Isogai A, et al. 2009. Fine-tuning of the cytoplasmic Ca²⁺ concentration is essential for pollen tube growth. *Plant Physiol* 150: 1322–1334.
- Johnson CH, Knight MR, Kondo T, Masson P, Sedbrook J, Haley A, Trewavas A. 1995. Circadian oscillations of cytosolic and chloroplastic free calcium in plants. *Science* 269: 1863–1865.
- Krebs M, Schumacher K. 2013. Live cell imaging of cytoplasmic and nuclear Ca²⁺ dynamics in *Arabidopsis* roots. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot073031.
- Krebs M, Held K, Binder A, Hashimoto K, Den Herder G, Parniske M, Kudla J, Schumacher K. 2012. FRET-based genetically encoded sensors allow high-resolution live cell imaging of Ca²⁺ dynamics. *Plant J* 69: 181–192.
- Knight H, Trewavas AJ, Knight MR. 1996. Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. *Plant cell* 8: 489–503.
- Knight MR, Campbell AK, Smith SM, Trewavas AJ. 1991. Transgenic plant aequorin reports the effect of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352: 524–526.
- Kudla J, Batistic O, Hashimoto K. 2010. Calcium signals: The lead currency of plant information processing. *Plant Cell* 22: 541–563.
- Logan DC, Knight MR. 2003. Mitochondrial and cytosolic calcium dynamics are differentially regulated in plants. *Plant Physiol* 133: 21–24.
- Loro G, Costa A. 2013. Imaging of mitochondrial and nuclear Ca²⁺ dynamics in *Arabidopsis* roots. *Cold Spring Harb Protoc* doi: 10.1101/pdb.prot073049.
- Loro G, Drago I, Pozzan T, Lo Schiavo F, Zottini M, Costa A. 2012. Targeting of Cameleon to different subcellular compartments reveals a strict cytoplasmic/mitochondrial Ca²⁺ handling relationship in plant cells. *Plant J* 71: 1–13.
- Mehlmer N, Parvin N, Hurst CH, Knight MR, Teige M, Vothknecht U. 2012. A toolset of aequorin expression vectors for *in planta* studies of subcellular calcium concentrations in *Arabidopsis thaliana*. *J Exp Bot* 63: 1751–1761.
- Monshausen GB, Messerli MA, Gilroy S. 2008. Imaging of the Yellow Cameleon 3.6 indicator reveals that elevations in cytosolic Ca²⁺ follow oscillating increases in growth in root hairs of *Arabidopsis*. *Plant Physiol* 147: 1690–1698.
- Monshausen G, Bibikova T, Weisenseel M, Gilroy S. 2009. Ca²⁺ regulates reactive oxygen species production and pH during mechanosensing in *Arabidopsis* roots. *Plant Cell* 21: 2341–2356.
- Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A. 2004. Expanded dynamic range of fluorescent indicators for Ca²⁺ by circularly permuted yellow fluorescent proteins. *Proc Natl Acad Sci* 101: 10554–10559.
- Palmer AE, Tsien RY. 2006. Measuring calcium signaling using genetically targetable fluorescent indicators. *Nat Protoc* 1: 1057–1065.
- Palmer AE, Giacomello M, Kortemme T, Hires S, Lev-Ram V, Baker D, Tsien RY. 2006. Ca²⁺ indicators based on computationally redesigned calmodulin–peptide pairs. *Chem Biol* 13: 521–530.
- Palmer AE, Qin Y, Park JG, McCombs JE. 2011. Design and application of genetically encoded biosensors. *Trends Biotechnol* 29: 144–152.
- Rincón-Zachary M, Teaster ND, Sparks JA, Valster AH, Motes CM, Blancaflor EB. 2010. Fluorescence resonance energy transfer-sensitized emission of Yellow Cameleon 3.60 reveals root zone-specific calcium signatures in *Arabidopsis* in response to aluminum and other trivalent cations. *Plant Physiol* 152: 1442–1458.
- Saito K, Hatsugai N, Horikawa K, Kobayashi K, Matsu-Ura T, Mikoshiba K, Nagai T. 2012. Auto-luminescent genetically-encoded ratiometric indicator for real-time Ca²⁺ imaging at the single cell level. *PLoS One* 5.
- Salinas J, Sanchez-Serrano JJ. 2006. Methods in molecular biology. *Arabidopsis protocols*, 2nd ed. Humana Press, New York, NY.
- Siebert B, Chabaud M, Timmers A, Monin A, Fournier J, Barker D. 2009. A nuclear-targeted Cameleon demonstrates intranuclear Ca²⁺ spiking in *Medicago truncatula* root hairs in response to rhizobial nodulation factors. *Plant Physiol* 151: 1197–1206.
- Tanaka K, Swanson S, Gilroy S, Stacey G. 2010. Extracellular nucleotides elicit cytosolic free calcium oscillations in *Arabidopsis*. *Plant Physiol* 154: 705–719.
- van Der Luit A, Olivari C, Haley A. 1999. Distinct calcium signaling pathways regulate calmodulin gene expression in tobacco. *Plant Physiol* 121: 705–714.
- Webb AR, McAinsh MR, Taylor JE, Hetherington AM. 1996. Calcium ions as intracellular second messengers in higher plants. *Adv Bot Res* 22: 45–96.
- Yang Y, Costa A, Leonhardt N, Siegel RS, Schroeder JI. 2008. Isolation of a strong *Arabidopsis* guard cell promoter and its potential as a research tool. *Plant Methods* 4: 6.

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