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Quantitative analysis of herbivore-induced cytosolic calcium by using a Cameleon (YC 3.6) calcium sensor in *Arabidopsis thaliana*

Francesca Verrillo[a, 1,] , Andrea Occhipinti[a, 1,] , Chidananda Nagamangala Kanchiswamy[b,] , Massimo E. Maffei[a,]

Abstract

Ca^{2+} is a key player in plant cell responses to biotic and abiotic stress. Owing to the central role of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) during early signaling and the need for precise determination of $[Ca^{2+}]_{cyt}$ variations, we used a Cameleon YC 3.6 reporter protein expressed in *Arabidopsis thaliana* to quantify $[Ca^{2+}]_{cyt}$ variations upon leaf mechanical damage (MD), herbivory by 3rd and 5th instar larvae of *Spodoptera littoralis* and *S. littoralis* oral secretions (OS) applied to MD. YC 3.6 allowed a clear distinction between MD and herbivory and discriminated between the two larvae instars. To our knowledge this is the first report of quantitative $[Ca^{2+}]_{cyt}$ determination upon herbivory using a Cameleon calcium sensor.

Keywords

- *Arabidopsis thaliana*;
- *Spodoptera littoralis*;
- Cameleon YC 3.6;
- Calcium signaling;
- Herbivory

Introduction

Calcium is as a key regulator of plant responses to endogenous stimuli and stress signals of both biotic and abiotic nature (Lecourieux et al., 2006, McAinsh and Pittman, 2009 and DeFalco et al., 2010). The variation of calcium concentration $[Ca^{2+}]$ at different subcellular localization, together with plasma V_m membrane depolarization and other second messengers (ROS, NOS and pH), are integrated in a signaling network that fine tunes and coordinates the downstream regulation of gene expression in response to stress (Maffei et al., 2006, Maffei et al., 2007a, Reddy et al., 2011 and Arimura et al., 2011).

In plants, cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) is maintained in the nM range (100–200 nM), whereas in many organelles and in the apoplast $[Ca^{2+}]$ reaches the mM range (Dodd et al., 2010). $[Ca^{2+}]_{cyt}$ can increase up to µM concentration in response to stress conditions (Messerli et al., 2000). This $[Ca^{2+}]_{cyt}$ variation is the result of a tight regulation of protein channels and transporters located in the plasma membrane and organelles' membranes (Jammes et al., 2011). $[Ca^{2+}]_{cyt}$ variations occur in the form of transients, spikes and oscillations (Lecourieux et al., 2006 and Kosuta et al., 2008) that mostly happen in a timeframe of seconds. The decoding of Ca^{2+} signal is controlled by several classes of Ca^{2+}-binding sensory proteins, including calmodulins, calmodulin-like proteins, calcineurin B-like proteins, and Ca^{2+}-dependent protein kinases (CPKs) (McAinsh and Pittman, 2009 and Dodd et al., 2010). Dynamics of Ca^{2+} spatial and temporal changes either in the cytosol and/or in other compartments of the plant cell are now accepted to generate "calcium signatures",

which might be responsible for the initiation of specific downstream events, eventually leading to appropriate responses (Mithöfer et al., 2009b, Batistic and Kudla, 2012 and Short et al., 2012).

Early events upon biotic stress, like that inflicted by insect herbivores, include an immediate and dramatic Ca^{2+} influx limited to a few cell layers lining the damage zone (Maffei et al., 2004, Maffei et al., 2007a, Arimura et al., 2008a and Wu and Baldwin, 2009). Several techniques have been used and developed to localize, measure and monitor $[Ca^{2+}]_{cyt}$ variations and a large number of fluorescent Ca^{2+} indicators are available for studying Ca^{2+} in plant cells (Haugland, 1996 and Mithöfer et al., 2009b). Besides bio-luminescent techniques using aequorin (Maffei et al., 2006), two fluorescent Ca^{2+} indicators have been used several times to successfully demonstrate the induction of Ca^{2+} signatures upon herbivory: Fluo-3 AM (Maffei et al., 2004, Maffei et al., 2006, Arimura et al., 2008a and Kanchiswamy et al., 2010) and Calcium Orange™ (Bricchi et al., 2010, Mohanta et al., 2012, Zebelo and Maffei, 2012, Zebelo et al., 2012 and Bricchi et al., 2013). Despite their proved efficacy, these two indicators do not allow a precise quantification of $[Ca^{2+}]_{cyt}$ variations. Another way to fine-tune Ca^{2+} variations is by using the Yellow Cameleon (YC) Ca^{2+}-sensor (Russell, 2011). Cameleons are genetically encoded fluorescent indicators based on cyan fluorescent protein (CFP), a C terminus of Calmodulin (CaM), a Gly–Gly linker, a CaM-binding domain of myosin light chain kinase (M13), and a yellow fluorescent protein (YFP) (Miyawaki et al., 1999 and Swanson et al., 2011). Binding of Ca^{2+} to CaM triggers a conformational change in the hinge portion that links the region between the CFP and YFP. The modification in proximity and orientation results in an enhanced fluorescence resonance energy transfer (FRET) efficiency between the two fluorophores. Changes in FRET efficiency between CFP and YFP are correlated with changes in $[Ca^{2+}]$ with a ratiometric relation (Swanson et al., 2011) (see also Supplementary Fig. S1).

Supplementary Fig. I.

Ratiometric spectral imaging measurement for the fluorescent protein calcium biosensor known as cameleon YC 3.6. The spectral profiles of YC 3.6 in the presence (red curve) and absence (yellow curve) of calcium demonstrate the high dynamic range of the probe at 530 nM. Cartoon drawings on the left of the cameleon biosensor are presented in the presence (b) and absence (c) of calcium. From http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/spectralfret.html.

Owing to the central role of early signaling through $[Ca^{2+}]_{cyt}$ variations in plant defense against herbivores, we used a YC 3.6 reporter protein expressed in *Arabidopsis thaliana* (Swanson and Gilroy, 2013) to fine-tune $[Ca^{2+}]_{cyt}$ variations in leaves upon mechanical damage (MD) and herbivory (HW). To induce Ca^{2+} signatures we used third (3rd HW) and fifth instar (5th HW) of *Spodoptera littoralis*, a model insect widely used to explore the activation and regulation of plant responses upon herbivory (Zebelo and Maffei, 2012; Zebelo et al., 2012 and Bricchi et al., 2012), as well as MD and MD plus application of *S. littoralis* oral secretions (OS).

Materials and methods

Plant and animal material

Arabidopsis thaliana L. (Columbia 0) plants were grown at 22 °C and 60% humidity from seed in plastic pots with sterilized potting soil held at 60% humidity, with daylight fluorescent tubes (120 μmol m^{-2} s^{-1}) and a photoperiod of 16 h. All experiments were carried out using 20–22-day-old plants (phases III of development (Boyes et al., 2001)). Seeds of *A. thaliana* YC 3.6 where kindly provided by Simon Gilroy (Department of Botany, University of Wisconsin, Madison, WI, USA) and plants were grown as above.

Spodoptera littoralis Boisd. (Lepidoptera, Noctuidae) larvae (kindly supplied as egg clutches by Syngenta, Switzerland) were fed on artificial diet (Bricchi et al., 2010) in Petri dishes (6 cm diameter) in a growth chamber with 16 h photoperiod at 25 °C and 60–70% humidity (Bricchi et al., 2013).

Plant defense responses were induced either by HW, repeated MD or MD plus application of *S. littoralis* OS which were collected as previously described (Zebelo and Maffei, 2012). Preliminary tests revealed no significant difference between OS from different larvae instars; therefore, OS were collected from 5th HW, which allowed to collect a higher amount of OS. Undamaged leaves were used as control. In order to compare the effect of HW with the action of the MD and MD + OS, we defined the timing of wounding at 30 min and we established a constant wounded leaf area, based on previous experiments (Bricchi et al., 2012), by adding a higher number of 3rd instar larvae.

Imaging of Cytosolic Ca^{2+} Levels by using FRET-Confocal laser scanning microscopy (CLSM)

Leaves of *A. thaliana* seedlings expressing FRET-based Ca^{2+} sensor YC 3.6 were ratio-imaged with a Nikon Eclipse C1 spectral CLS microscope using 20× and 60× oil-immersion objectives. The YC 3.6 Ca^{2+} sensor was excited with a 458-nm wavelength by using an argon laser. The cyan fluorescent protein (CFP; 473–505 nm) and FRET-dependent Venus (526–536 nm) emission were assayed using a krypton/argon laser at 488 nm with a BP of 500–540 nm and a LP of 650 nm. In situ calibration was performed by rising Ca^{2+} to saturating levels for YC 3.6. Cells were permeabilized to allow a massive free diffusion of calcium inside the cell to get the R_{max}. The trapping of free calcium released from the cells was performed by EGTA. The maximum FRET/CFP ratio was obtained by treatment with 1 M $CaCl_2$ in response to mechanical perturbation (R_{max} = 0.690). The minimum FRET/CFP ratio (R_{min} = 0.309) was recorded by treatment with 1 M Tris 100 mM EDTA and 50 mM EGTA solution. Control, HW, MD and MD + OS $[Ca^{2+}]_{cyt}$ variations were then calculated according to the equation:

$$Ca^{2+} = K_d \frac{(R - R_{min})}{(R_{max} - R)^{1/n}}$$ Ca2+=Kd(R−Rmin)(Rmax−R)1/n

where R represents the FRET/CFP ratio measured during the experiment, n the Hill coefficient that was 1 for YC 3.6, while K_d values were assessed for Ca^{2+} = 250 nM (Monshausen et al., 2008). Measurements were repeated at least five times (biological replicates) and were performed at about 0.5–1.5 mm from the wounded zone after 30 min from treatment. For OS experiments, 5 μl OS were applied as previously described (Zebelo and Maffei, 2012). The images presented are the result of the accumulation of calcium signals.

Statistical analysis

The obtained data were processed by using the stem-and-leaf function of Systat 10 in order to calculate the lower and upper hinge from the Gaussian distribution of values. Data were then filtered and the mean value was calculated along with the Standard Error (SE) (Bricchi et al., 2010). Paired t test Dunn–Sidak and Bonferroni adjusted probability were used to assess the difference between treatments and the control ($P < 0.05$). For all experiments, at least five samples per treatment were used. Data are expressed as mean values ± standard error.

Results and discussion

Variations in $[Ca^{2+}]_{cyt}$ levels mediate the plant signal transduction pathways, eventually leading to both direct and indirect responses (Maffei et al., 2007b, Mazars et al., 2009, Mithöfer et al., 2009b and War et al., 2012). Since the herbivore-dependent Ca^{2+} variation is decoded by signaling cascades (Arimura et al., 2000 and Kanchiswamy et al., 2010), the investigation of physiological $[Ca^{2+}]_{cyt}$ changes is instrumental to dissect the signaling pathway upon herbivore damage. The results of the present work add novel facets to what previously known regarding the Ca^{2+} signaling upon chewing insect feeding. Here we show that Arabidopsis plants stably transformed with a cytosolic version of the GFP-based Ca^{2+} sensor Yellow Cameleon (YC 3.6) (Monshausen et al., 2008), allowed quantification of $[Ca^{2+}]_{cyt}$ changes upon MD and HW. YC 3.6 showed a high sensitivity for HW responses and a high specificity for subcellular cytosolic Ca^{2+} quantification, as expected (Monshausen et al., 2008, Loro et al., 2012 and Krebs et al., 2012).

One of the major challenges in detecting $[Ca^{2+}]_{cyt}$ variations upon herbivory is the assessment of basal level of the ion and the possible effects of MD, an abiotic stress that occurs during HW (Maffei et al., 2004). Therefore, we first measured basal $[Ca^{2+}]_{cyt}$ variations in undamaged (control) Arabidopsis YC 3.6 leaves and we found a FRET corresponding to about 80 nM $[Ca^{2+}]_{cyt}$. High CLSM magnification offered details on the subcellular cytosolic localization of this basal FRET (Fig. 1). MD prompted a significant increase of FRET with $[Ca^{2+}]_{cyt}$ levels of about 160 nM. As expected, in both control and MD leaves a cytosolic Ca^{2+} localization was confirmed.

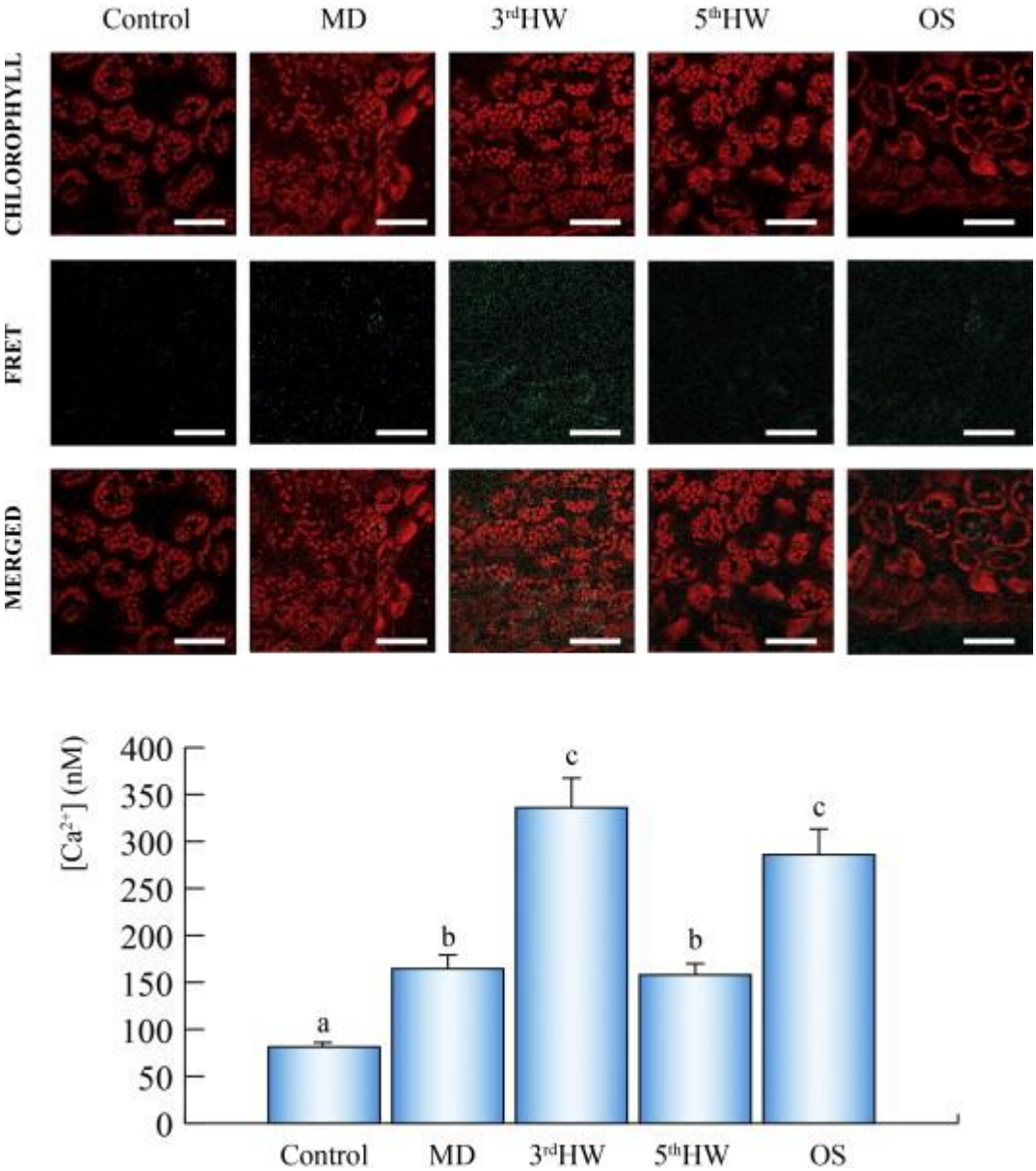

Fig. 1.

Intracellular cytosolic calcium variations in *Arabidopsis thaliana* leaves expressing a Yellow Cameleon (YC3.6) reporter protein upon different treatments. False-color image analysis reconstructions from confocal laser-scanning microscope observations, and fluochemical intracellular Ca^{2+} determination and image analysis. Pictures represent portions of the Arabidopsis leaf blade where the green fluorescence refers to FRET signal whereas the chloroplasts are evidenced by a bright red color caused by chlorophyll fluorescence. Quantitative analysis of the Ca^{2+} release shown in confocal figures is shown in the bottom graph. Error bars represent standard error. Different letters indicate significant differences ($P < 0.05$, Tukey–Kramer HSD). Scale bar (50 μM) is indicated on the figures. Control, undamaged leaves; MD, mechanical damage; 3rd HW, herbivore damage caused by 3rd instar *Spodoptera littoralis* larvae; 5th HW, herbivore damage caused by 5th instar *S. littoralis* larvae; OS, application of *S. littoralis* oral secretions to MD.

Having assessed the basal levels, we evaluated $[Ca^{2+}]_{cyt}$ levels in leaves subjected to *S. littoralis* herbivory. It was demonstrated that different instars may induce different responses in fed plants (Mithöfer et al., 2009a); however, no significant differences were observed in $[Ca^{2+}]_{cyt}$ levels from

OS extracted from either the third (3rd HW) or fifth instar (5th HW) larvae (data not shown). Nevertheless, we treated Arabidopsis YC 3.6 leaves with the two different instars. A significant increase of $[Ca^{2+}]_{cyt}$ levels was found with respect to control and MD leaves when leaves were fed by 3rd HW, with values above 330 nM; however, feeding by 5th HW lowered the $[Ca^{2+}]_{cyt}$ levels to MD values (about 150 nM). 3rd HW- and 5th HW-dependent cytosolic Ca^{2+} localization was confirmed (Fig. 1). It was interesting to note that 3rd HW was able to induce a stronger Ca^{2+} response with respect to 5th HW, despite the same leaf area damage inflicted to Arabidopsis. Third instar larvae of *S. littoralis* are frequently used for plant responses to herbivory because they induce significant responses in fed plants (Arimura et al., 2008b), in this work we could demonstrate that these responses depend on a significantly higher $[Ca^{2+}]_{cyt}$ induction in fed plant tissues. Since we can exclude a different contribution of OS between the instars, we argue that the observed effect might depend on a higher quantity of OS secreted by 3rd HW. In fact, in order to obtain the same leaf area damage within 30 min, a higher number of larvae had to be applied.

It is known that application of *S. littoralis* OS on MD leaves induces responses that are comparable to HW (Zebelo and Maffei, 2012). We therefore quantified $[Ca^{2+}]_{cyt}$ levels in Arabidopsis YC 3.6 leaves by applying 5th HW OS to MD. The plant response was a significant increase of $[Ca^{2+}]_{cyt}$ with values (~290 nM) not significantly different to those obtained upon 3rd HW, but significantly higher that all other treatments (Fig. 1). Previous results have demonstrated that the level of leaf response to OS is proportional to the quantity of OS (Zebelo and Maffei, 2012; Bricchi et al., 2013).

The use of in situ calibration curves is a reliable method to calculate the apparent Ca^{2+}-binding affinity (K_d) and minimum and maximum fluorescence level (R_{min} and R_{max})(Swanson et al., 2011).

Many comparisons between the Ca^{2+}-reporter protein Cameleon and other fluorescent/luminescent based Ca^{2+} probes have been reported so far. To our knowledge, this is the first time that a Cameleon construct is used to quantify $[Ca^{2+}]_{cyt}$ changes upon herbivory and insect's oral secretions. Our results confirm the higher specify and sensitivity in the detection of $[Ca^{2+}]_{cyt}$ changes for Cameleon reporter protein and provide quantitative estimates of $[Ca^{2+}]_{cyt}$ variations upon herbivory.

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