Analyses of Ca\textsuperscript{2+} Accumulation and Dynamics in the Endoplasmic Reticulum of Arabidopsis Root Cells Using a Genetically Encoded Cameleon Sensor\textsuperscript{1[C][W]}

Maria Cristina Bonza\textsuperscript{2}, Giovanna Loro\textsuperscript{2}, Smrutisanjita Behera, Andrea Wong, Jörg Kudla, and Alex Costa\textsuperscript{*}

Department of Biosciences, University of Milan, 20133 Milan, Italy (M.C.B., G.L., A.C.); Department of Biology (G.L.) and Department of Biomedical Sciences (A.W.), University of Padua, 35131 Padova, Italy; Institut für Biologie und Biotechnologie der Pflanzen, Universität Münster, 48149 Münster, Germany (S.B., J.K.); and Institute of Biophysics, Consiglio Nazionale delle Ricerche, 20133 Milan, Italy (A.C.)

In planta, very limited information is available about how the endoplasmic reticulum (ER) contributes to cellular Ca\textsuperscript{2+} dynamics and homeostasis. Here, we report the generation of an ER-targeted Cameleon reporter protein suitable for analysis of Ca\textsuperscript{2+} in the ER. We show that the luminal ER Ca\textsuperscript{2+} elevations typically follow cytosolic ones, but with distinct dynamics. These differences are fundamental for the function of the ER in cellular Ca\textsuperscript{2+} homeostasis in plants and animals.

In plants, rises in cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) occur in response to both biotic and abiotic stimuli (Hetherington and Brownlee, 2004; McAinsh and Pittman, 2009; Kudla et al., 2010; Bose et al., 2011). Depending on the stimulus, these rises can display the form of a single transient or repetitive Ca\textsuperscript{2+} oscillations and are commonly designated as “Ca\textsuperscript{2+} signatures” (Webb et al., 1996; Allen et al., 2000, 2001; Sanders et al., 2002; Young et al., 2006; Kudla et al., 2010).

The generation and shaping of [Ca\textsuperscript{2+}]\textsubscript{cyt} signatures depends on fine-tuning of Ca\textsuperscript{2+} influxes and effluxes occurring at both the plasma membrane (PM) and membranes of the different subcellular compartments (Pittman and Hirschi, 2003; Hetherington and Brownlee, 2004; Dodd et al., 2010; Spalding and Harper, 2011). The opening of Ca\textsuperscript{2+}-permeable influx channels in response to a stimulus will release Ca\textsuperscript{2+} into the cytosol and cause the generation of a Ca\textsuperscript{2+} spike, while the activity of Ca\textsuperscript{2+} efflux transporters (H+-Ca\textsuperscript{2+} antiporters and Ca\textsuperscript{2+}-ATPases) will return the [Ca\textsuperscript{2+}]\textsubscript{cyt} to resting concentrations (McAinsh and Pittman, 2009; Bonza and De Michielis, 2011; Spalding and Harper, 2011).

Recently, the development and application of genetically encoded Ca\textsuperscript{2+} reporter proteins like Cameleons has allowed the study of Ca\textsuperscript{2+} dynamics in several compartments with organ, tissue, and single-cell resolution (Allen et al., 1999; Monshausen et al., 2008; Yang et al., 2008; Sieberer et al., 2009; Costa et al., 2010; Rincón-Zachary et al., 2010; Tanaka et al., 2010; Michard et al., 2011; Krebs et al., 2012; Loro et al., 2012; Behera et al., 2013). Cameleons are fluorescence resonance energy transfer (FRET)-based indicators in which two GFP variants, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), are linked together by the Ca\textsuperscript{2+}-binding protein calmodulin (CaM) and a CaM-binding peptide (Miyawaki et al., 1997). Binding of Ca\textsuperscript{2+} to the Ca\textsuperscript{2+}-responsive elements alters the efficiency of FRET, allowing for quantitative measurements of Ca\textsuperscript{2+} dynamics. Different Cameleon variants have been developed since the first report about this reporter protein (Miyawaki et al., 1997). This involved, for example, advanced versions with improved fluorescence, larger changes in FRET upon Ca\textsuperscript{2+} binding, and a broad range of Ca\textsuperscript{2+} affinities (Palmer and Tsien, 2006). Moreover, mutational modification of the original Cameleon in the case of the

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\textsuperscript{2}These authors contributed equally to the article.

\textsuperscript{*}Address correspondence to alex.costa@unimi.it.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Alex Costa (alex.costa@unimi.it).

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D family of indicators ensured that their function is no longer perturbed by large excesses of native CaM (Palmer et al., 2006). The use of Cameleons in plants has allowed the study of cytosolic (Krebs et al., 2012), nuclear (Sieberer et al., 2009; Krebs et al., 2012), peroxisomal (Costa et al., 2010, 2013), and mitochondrial (Loro et al., 2012, 2013) Ca\(^{2+}\) dynamics in specific plant organs and single cells. However, despite all these advances, very limited data have been reported regarding in vivo analyses of endoplasmic reticulum (ER) Ca\(^{2+}\) dynamics in plants (Iwano et al., 2009).

In animal cells, the ER represents an important Ca\(^{2+}\) storage organelle in which the free Ca\(^{2+}\) concentration varies between 50 and 500 \(\mu M\) (Coe and Michalak, 2009). Ca\(^{2+}\) release from the ER is involved in many different processes, including exocytosis, contraction, metabolism, regulation of transcription, fertilization, and apoptosis. The major Ca\(^{2+}\) entry pathway in electrically nonexcitable cells is represented by the “store-operated Ca\(^{2+}\) entry” (SOCE; Feske et al., 2012). Here, PM-localized calcium release-activated channels are activated in response to the emptying of intracellular ER Ca\(^{2+}\) stores (Parekh and Putney, 2005; Carrasco and Meyer, 2010). Moreover, in several mammalian cell types upon stimulation, Ca\(^{2+}\) is released from the ER into the cytosol through the activity of different classes of ER-resident Ca\(^{2+}\)-permeable channels activated by second messengers such as inositol 1,4,5-trisphosphate (IP3) and cyclic adenosine diphosphoribose (Parekh and Putney, 2005; Berridge, 2009; Galiano and Chuang, 2012). In sharp contrast, for plant cells, no precise data on ER Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{ER}}\)) are available (Stael et al., 2012), and in vivo investigations about the Ca\(^{2+}\) storage properties of the ER have remained very limited (Iwano et al., 2009).

Moreover, SOCE has not been reported in plants, and stromal interaction molecule (STIM) proteins, which are central components of SOCE, are not encoded in the genomes of higher plants (Collins and Meyer, 2011). Despite some biochemical evidence for Ca\(^{2+}\) release in response to IP3, nicotinic acid adenine dinucleotide phosphate, and cyclic adenosine diphosphoribose (Muir and Sanders, 1997; Navazio et al., 2000, 2001), electrophysiological analyses supporting the existence of voltage-gated ER-localized Ca\(^{2+}\) channels (Klisenen et al., 1995, 1997), homologous for the respective IP3 and ryanodine receptors, are missing in higher plants (Wheeler and Brownlee, 2008; Kudla et al., 2010). All considered, this situation suggests that the role of the ER for cellular Ca\(^{2+}\) dynamics may be fundamentally different in plants as compared with animal cells.

Plants contain two major types of Ca\(^{2+}\) pumps named ECA (for ER-type Ca\(^{2+}\)-ATPase) and ACA (for autoinhibited Ca\(^{2+}\)-ATPase; Geisler et al., 2000; Sze et al., 2000; Bonza and De Michielis, 2011). Plant genomes encode different isoforms of both ECAs and ACAs. These are often coexpressed in certain cell types, and consequently, different cellular membranes in the same cell contain multiple Ca\(^{2+}\)-ATPase isoforms (Bonza and De Michielis, 2011). In Arabidopsis (Arabidopsis thaliana), immunological and membrane fractionation studies provided evidence for an ER localization of at least two distinct Ca\(^{2+}\) pumps, ECA1 and ACA2 (Li et al., 1997; Harper et al., 1998; Liang and Sze, 1998; Hong et al., 1999; Hwang et al., 2000). Moreover, additional ECA and ACA isoforms are predicted to be ER localized (Geisler et al., 2000; Sze et al., 2000, Baxter et al., 2003; Bonza and De Michielis, 2011). Biochemical characterization revealed that ECA1 and ACA2, besides different regulatory properties, also have different affinities for Ca\(^{2+}\), with one-half saturation concentration for free Ca\(^{2+}\) in the submicromolar and micromolar range, respectively (Liang and Sze, 1998; Hwang et al., 2000; Sze et al., 2000; Wu et al., 2002; Bonza and De Michielis, 2011), suggesting their participation in different aspects of ER Ca\(^{2+}\) regulation. The main predicted function for the ER Ca\(^{2+}\)-ATPases is Ca\(^{2+}\) loading into the ER lumen (Corbett and Michalak, 2000; Persson and Harper, 2006). However, how exactly these ER Ca\(^{2+}\)-ATPases contribute to ER and cytosolic Ca\(^{2+}\) dynamics awaits further investigation.

RESULTS AND DISCUSSION
Generation of a Cameleon-Based Reporter Protein for Monitoring Ca\(^{2+}\) Dynamics in the ER of Plants

In order to enable analyses of Ca\(^{2+}\) dynamics in the ER of plant cells, we first took advantage of the available D1ER Cameleon Ca\(^{2+}\) reporter protein that had already been successfully used to study Ca\(^{2+}\) dynamics in the ER of mammalian cells (Palmer et al., 2004; Luciani et al., 2009; Jiménez-Moreno et al., 2010). In this reporter construct, fusion of the D1 reporter protein with a mammalian ER targeting signal, allowed us to evaluate in vivo the contribution of the different Ca\(^{2+}\)-ATPase classes in [Ca\(^{2+}\)]\(_{\text{ER}}\) homeostasis. Taken together, our data do not support a role of the ER as a major Ca\(^{2+}\)-releasing source for stimulus-induced increases in cytosolic Ca\(^{2+}\) concentration. Instead, our results show that the luminal ER Ca\(^{2+}\) transients typically follow cytosolic increases, but with distinct dynamics. These findings suggest fundamental differences for the function of the ER in cellular Ca\(^{2+}\) homeostasis in plants and animals.
Since D1 Cameleon has a biphasic Ca\(^{2+}\) dependency in vitro (dissociation constant = 0.8 and 60 \(\mu M\); Palmer and Tsien, 2006), we supposed that the resting \([Ca^{2+}]_{\text{ER}}\) in the analyzed cells was above these values. Therefore, we decided to evaluate an alternative probe with a different affinity for Ca\(^{2+}\). The Cameleon D4 variant has an in vitro dissociation constant for Ca\(^{2+}\) of 195 \(\mu M\) (Palmer et al., 2006) and was very recently used by two groups to monitor free Ca\(^{2+}\) levels in the ER lumen of mammalian cells (Ravier et al., 2011; Kipanyula et al., 2012). To efficiently target the D4 probe to the lumen of plant cell ER, we followed the same cloning strategy adopted for the production of the CRT-D1ER construct. The Arabidopsis CALRETICULINA (Christensen et al., 2010) signal peptide was fused upstream of the D4ER Cameleon reporter (Kipanyula et al., 2012), and the entire coding sequence was placed under the control of a single cauliflower mosaic virus (CaMV) 35S promoter. The resulting construct, CRT-D4ER (Fig. 1), was transiently expressed in tobacco (Nicotiana benthamiana) leaf epidermal cells (Fig. 1, A–C). Comparison with the localization of nWAK2-mCherry-HDEL (for a full description of the construct, see Nelson et al., 2007) as an ER marker that was coexpressed in these cells revealed that nWAK2-mCherry-HDEL and CRT-D4ER fluoresces fully merged in the nuclear envelope of tobacco epidermal cells (Fig. 1, C and D). The CRT-D4ER reporter construct was subsequently transformed into Arabidopsis plants, and 15 independent stable transgenic lines were selected. Figure 1, E to H, provides representative images of CRT-D4ER Arabidopsis transgenic plants, demonstrating that the probe was expressed in root (Fig. 1E), hypocotyl (Fig. 1F), and mature leaf cells (Fig. 1G). In these different tissues/organs, the typical ER morphology was clearly recognized (Boevink et al., 1999; Brandizzi et al., 2002), with 1- to 5- \(\mu M\) ER fusive bodies (Hawes et al., 2001; Matsushima et al., 2002, 2003; Nelson et al., 2007) detected in the hypocotyl cells (Fig. 1G). The CRT-D4ER probe was also abundantly expressed in guard cells (Fig. 1, G and H). In order to investigate the Ca\(^{2+}\) reporter protein conformation status in resting conditions, we imaged the root tip of transgenic CRT-D4ER seedlings by means of a wide-field fluorescence microscope (excited with a 436/20-nm light wavelength) for the simultaneous acquisition of the CFP and YFP emission wavelengths. In root tip cells expressing CRT-D4ER, the fluorescences of both CFP and the FRET acceptor (YFP in the D4) were detected and were clearly above the level of organ autofluorescence (Fig. 1, I and J). Similar results were obtained by confocal microscopic analysis performed both in leaves and root cells (data not shown).

### CRT-D4ER Allows the Visualization of ER Ca\(^{2+}\) Dynamics in Root Cells

Subsequently, we sought to use CRT-D4ER to visualize potential variations in \([Ca^{2+}]_{\text{ER}}\) in response to defined stimuli. To this end, we challenged 7-d-old Arabidopsis seedlings with different stimuli reported to induce cytosolic Ca\(^{2+}\) rises in root cells, such as external ATP (Tanaka et al., 2010; Loro et al., 2012, 2013), i-Glu (Qi et al., 2006), and NaCl (Kiegle et al., 2000). To allow for comparison with cytosolic Ca\(^{2+}\) dynamics and as a control for the efficiency of stimulus applications, we performed a series of parallel experiments using either seedlings expressing the cytosol-localized Cameleon YC3.6 (NES-YC3.6; Nagai et al., 2004; Krebs et al., 2012) or expressing CRT-D4ER (Fig. 2).

One millimolar i-Glu was able to stimulate a fast and transient \([Ca^{2+}]_{\text{cyt}}\) increase in root tip cells (Fig. 2, A and G). In particular, i-Glu triggered a response in the cells of the root tip region, and then Ca\(^{2+}\) elevations spread out quickly to the upper root tip cells (Fig. 2A; Supplemental Movies S1 and S2). The i-Glu-Ca\(^{2+}\)-induced elevation is probably dependent on the activity of members of the ionotropic Glu receptors, channels that have been demonstrated to facilitate Ca\(^{2+}\) influx across the PM (Michard et al., 2011; Vincill et al., 2012, 2013). Several members of this large family (20 members in Arabidopsis; Lacombe et al., 2001) are in fact expressed in the different tissues of root tip, as reported by published microarray and experimental data (Winter et al., 2007; Vincill et al., 2013).

The same stimulus also triggered a concomitant increase of \([Ca^{2+}]_{\text{ER}}\) (Fig. 2, B and G), which was more sustained compared with the rise observed in the cytosol (Fig. 2, B and H). Importantly, separate analyses of the two CFP and FRET fluoresces revealed an evident FRET response, manifested by a decrease of CFP and an increase of YFP emissions (Supplemental Fig. S2A).

A similar series of experiments was then performed with the addition of 0.5 \(mM\) ATP (Fig. 2, C and D). In agreement with previous reports, high extracellular ATP concentration resulted in a strong and sustained \([Ca^{2+}]_{\text{cyt}}\) increase, with a typical dynamics consisting of different sequential Ca\(^{2+}\) peaks due to the influx of external Ca\(^{2+}\) and the release of Ca\(^{2+}\) from nonidentified internal stores (Fig. 2C; Supplemental Movie S3; Tanaka et al., 2010; Loro et al., 2012). This stimulus also induced an ER Ca\(^{2+}\) accumulation (Fig. 2D) in which the maximum level of \([Ca^{2+}]_{\text{ER}}\) was reached later as compared with the cytosol (Fig. 2G) but was stronger and even more sustained as compared with the one induced by i-Glu (Fig. 2, D, H, and I). In none of these experiments did we observe a measurable reduction in \([Ca^{2+}]_{\text{ER}}\) before the occurrence of the \([Ca^{2+}]_{\text{cyt}}\) increase. To further address this aspect, a series of experiments was performed with 0.01 mM ATP, a concentration reported to stimulate ER Ca\(^{2+}\) release in HeLa cells (Palmer et al., 2004). As reported previously, root tip cells stimulated with 0.01 mM ATP displayed \([Ca^{2+}]_{\text{cyt}}\) dynamics, albeit of lower magnitude (Supplemental Fig. S3, A and C–E; Loro et al., 2012). However, again, only an increase in \([Ca^{2+}]_{\text{ER}}\) was observed (Supplemental Fig. S3, B–E). These results strongly support the conclusion that the ER does not represent the internal Ca\(^{2+}\)-releasing store involved in the generation of the ATP-induced cytosolic...
Ca$^{2+}$ dynamics. Finally, salt stress was analyzed as a third stimulus that is known to elicit an increase in cytosolic Ca$^{2+}$ in root cells. The effect of NaCl was analyzed in the root mature zone, since these cells respond in a more pronounced manner than root tip cells to salt stress (data not shown). The addition of 100 mM NaCl induced a fast cytosolic Ca$^{2+}$ increase (Fig. 2, E and G), which was accompanied by a Ca$^{2+}$ accumulation in the ER (Fig. 2, F, H, and I). In the latter case, the source of cytosolic Ca$^{2+}$ has been demonstrated to be both from extracellular and intracellular calcium stores, mainly from vacuole (Knight et al., 1997). Our results indeed demonstrate that the ER is not part of such intracellular stores. Moreover, it is worth noting that, compared with the response to l-Glu (Fig. 2, G–I), the increases in [Ca$^{2+}$]$_{cyt}$ were similar in terms of peak intensities and duration, while the ER Ca$^{2+}$ accumulation induced by NaCl treatment was smaller. This observation could be related to the fact that cells of the root mature zone have extensive vacuoles compared with the meristematic cells of the root tip, hence probably also contributing to Ca$^{2+}$ sequestration (Peiter, 2011).

Altogether, these results indicate that plant cells transiently accumulate Ca$^{2+}$ in the ER in response to different stimuli, similar to what was observed in the apoplast and mitochondria (Gao et al., 2004; Loro et al., 2012). The subsequent decrease in [Ca$^{2+}$]$_{ER}$, which is especially evident with l-Glu, low ATP concentration, and NaCl treatments, occurs only after the initial accumulation and is likely to be dependent on the activity of ER membrane-resident Ca$^{2+}$-permeable channels (Klüsener et al., 1995, 1997; Muir and Sanders, 1997; Navazio et al., 2000, 2001). Importantly, these results suggest that, at least in the cases investigated here, the ER does not contribute to the generation of the observed cytosolic Ca$^{2+}$ increases by releasing Ca$^{2+}$. Instead, it appears that the ER represents a system that mimics [Ca$^{2+}$]$_{cyt}$ increases, but with distinct dynamics, as marked by the different times at which the peaks of [Ca$^{2+}$]$_{cyt}$ and [Ca$^{2+}$]$_{ER}$ are reached after the stimuli perception (Fig. 2G). The delayed ER Ca$^{2+}$ accumulation was particularly evident in ATP-stimulated cells (Fig. 2H; Supplemental Fig. S3E). These dynamics of ER Ca$^{2+}$ accumulation may reflect the intrinsic properties of the ER.

C. The YFP signal of CRT-D4ER colocalizes with mCherry, as revealed by colocalization of the two fluorescence signals at the level of the nuclear envelope (NE). D. Plot profile of YFP and mCherry fluorescence corresponding to the marked white line shown in C. E to H, Confocal microscopy analyses revealed efficient CRT-D4ER YFP expression in different organelles of Arabidopsis stable transgenic plants with a subcellular distribution showing typical ER features. E, Root tip cells. F, Hypocotyl cells, showing the presence of fusiform bodies. G, Three-dimensional maximum projection of a mature leaf region. H, Stomata guard cells. I and J, Representative root tip of a CRT-D4ER seedling excited with 440-nm light for FRET detection. I, CFP emission. J, FRET emission. The simultaneous detection of the two emissions demonstrates that CFP was not quenched and YFP was properly excited through FRET.
of the ER-resident active Ca\(_{\text{ER}}\) transport systems (Bonza and De Michelis, 2011; Bose et al., 2011). Instead, the \([\text{Ca}^{2+}]_{\text{cyt}}\) increase is mainly dependent on the activity of channels occurring at very much faster rate than transporters.

To corroborate that Ca\(_2^+\) accumulation in the ER mimics cytosolic Ca\(_2^+\) rises, we performed a series of experiments in which two different ATP concentrations were applied successively (0.01 mM followed by 0.5 mM) to root tip cells (Fig. 3). In particular, the second administration was applied before both \([\text{Ca}^{2+}]_{\text{cyt}}\) and \([\text{Ca}^{2+}]_{\text{ER}}\) were completely recovered to resting values. Figure 3A reports the superimposition of representative ratio images of the experiment shown in the corresponding trace above. A, i-Glu (1 mM) induced a single steep and fast Ca\(_2^+\) transient in the cytosol of root tip cells. B, the same stimulus of A induced an ER Ca\(_2^+\) accumulation showing a slow kinetic and a sustained Ca\(_2^+\) recovery phase. C, ATP (0.5 mM) induced a strong cytosolic Ca\(_2^+\) transient in the cytosol of root tip cells, where different peaks were recognizable. The recovery phase was more sustained compared with 1 mM i-Glu. D, the same stimulus of C induced an ER Ca\(_2^+\) accumulation showing a slow kinetic, but with values about four times higher than those observed with 1 mM i-Glu and, afterward, with a small \([\text{Ca}^{2+}]_{\text{ER}}\) decrease. E, NaCl (100 mM) induced a biphasic cytosolic Ca\(_2^+\) response with two peaks of different intensities in cells of the root mature zone. F, the same salt concentration of E led to a small ER Ca\(_2^+\) accumulation followed by a slow \([\text{Ca}^{2+}]_{\text{ER}}\) decrease. G, Statistical analysis of times at which the maximum \(\Delta R/R_0\) variations were measured in both compartments in response to different applied stimuli. H, Statistical analysis of \(\Delta R/R_0\) variations measured in both compartments 120 s after sensing of the different applied stimuli. The inset shows a magnification of i-Glu Ca\(_2^+\) peak averages. I, Statistical analysis of maximum \(\Delta R/R_0\) variations measured in both compartments in response to different applied stimuli. \(P\) values were calculated using Student’s t test. Bars = 100 \(\mu\)m. [See online article for color version of this figure.]
buffering the cytosolic Ca\(^{2+}\) rises. Together, these results indicate that Ca\(^{2+}\) accumulation in the ER is strictly dependent on the [Ca\(^{2+}\)]\(_{\text{cyt}}\). Finally, when root cells were challenged with a series of hyperpolarizing-depolarizing buffers, known to induce repetitive cytosolic Ca\(^{2+}\) transients in guard and root cells (Allen et al., 2000, 2001; Weinl et al., 2008; Krebs et al., 2012), repetitive ER Ca\(^{2+}\) accumulations were also observed (Supplemental Fig. S4, A and B). Chelating extracellular Ca\(^{2+}\) with EGTA prevented both cytosolic and ER Ca\(^{2+}\) transients (Supplemental Fig. S4, C and D), confirming that the ER does not contribute as an intracellular Ca\(^{2+}\) store to the generation of stimulus-induced cytosolic Ca\(^{2+}\) rises observed. However, we can currently not fully exclude that localized Ca\(^{2+}\) releases from ER occur in specific microdomains of cells (Rizzuto and Pozzan, 2006). Based on our microscope resolution, the predominant response of a defined population of root tip cells was measurable that consequently represents the predominant component of ER Ca\(^{2+}\) dynamics in these cells.

Comparison of ER Ca\(^{2+}\) dynamics induced in root tip cells by l-Glu and ATP revealed that Ca\(^{2+}\) accumulation in the ER was directly dependent on the magnitude of cytosolic Ca\(^{2+}\) elevation, with ATP inducing the strongest increase (compare the AR/R\(_{0}\) in Fig. 2, B, D, and I). In particular, the observed differences between the responses of root tip cells to l-Glu and ATP may result from a different number of cells responding primarily or secondarily to the stimulus (see the image sequences below the graphs in Fig. 2). In each single cell, the two stimuli may produce an increase in [Ca\(^{2+}\)]\(_{\text{ER}}\) of different amplitude. In order to appreciate Ca\(^{2+}\) dynamics in single cells, we performed an analysis of CRT-D4ER-expressing seedlings by means of confocal microscopy. The results, depicted in Figure 4 and Supplemental Movies S4 and S5, demonstrated that amplitudes and dynamics of ER Ca\(^{2+}\) transients induced by l-Glu and ATP in single cells essentially reflected the general root tip response. Importantly, these results confirm that our wide-field analyses faithfully reflect the cellular responsiveness of root tip cells to the stimuli investigated.

Altogether, these experiments demonstrate that the CRT-D4ER represents a reliable probe to monitor ER Ca\(^{2+}\) dynamics in vivo at both the organ and single-cell levels. Moreover, our results suggest that, in the cells investigated under the conditions examined here, the ER does not represent a major store for releasing Ca\(^{2+}\) into the cytosol.

**ER-Localized Ca\(^{2+}\)-ATPases Modulate ER Ca\(^{2+}\) Homeostasis**

Having established that dynamic changes in Ca\(^{2+}\) accumulation occur in the ER in response to defined stimuli, we next aimed at elucidating the contribution of ER-resident Ca\(^{2+}\)-ATPases to [Ca\(^{2+}\)]\(_{\text{ER}}\) homeostasis. In Arabidopsis, members of both classes of Ca\(^{2+}\) pumps are resident in the ER membrane (Sze et al., 2000; Bonza and De Michelis, 2011). ECAs are specifically inhibited by cyclopiazonic acid (CPA; Liang and Sze, 1998), while ACAs are particularly sensitive to inhibition by fluorescein derivatives such as erythrosin B or eosin Y (Eos; De Michielis et al., 1993; Geisler et al., 2000; Sze et al., 2000; Bonza et al., 2004; Bonza and De Michielis, 2011). Therefore, in order to evaluate the contribution of the different types of Ca\(^{2+}\)-ATPases to ER Ca\(^{2+}\) homeostasis, a pharmacological approach was pursued. Published microarray data (Winter et al., 2007) confirmed that root tips of young Arabidopsis seedlings express Ca\(^{2+}\) pumps
of both types at various cellular membranes, including the ER-localized Ca\(^{2+}\)-ATPase isoforms ACA2 and ECA1 (data not shown).

Arabidopsis seedlings expressing the CRT-D4ER or NES-YC3.6 Cameleon reporter protein were incubated for 10 or 60 min with or without 25 \(\mu\)M CPA, 0.5 \(\mu\)M Eos, and 25 \(\mu\)M CPA plus 0.5 \(\mu\)M Eos. In order to evaluate the [Ca\(^{2+}\)]\(_{\text{ER}}\) and [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels in the different tested conditions, root tips were then analyzed by measuring the ratio values for both CRT-D4ER and NES-YC3.6 probes (Fig. 5). Root tip cells were chosen for the analysis because they harbor smaller vacuoles compared with cells of the mature zone. In this way, we probably reduced the relative contribution of the vacuolar Ca\(^{2+}\) transport systems to the regulation of cytosolic Ca\(^{2+}\) homeostasis (Cheng et al., 2005; Conn et al., 2011; Peiter, 2011).

The ECA inhibitor CPA affected the CRT-D4ER ratio already after 10 min of treatment, resulting in a significant decrease in the [Ca\(^{2+}\)]\(_{\text{ER}}\) compared with the control condition (Fig. 5A, gray bar). Surprisingly, treatment with the ACA inhibitor Eos did not lead to a [Ca\(^{2+}\)]\(_{\text{ER}}\) decrease but to a [Ca\(^{2+}\)]\(_{\text{ER}}\) increase (Fig. 5A, white bar). Interestingly, when treatments were performed with both inhibitors simultaneously (Fig. 5A, striped bar), the [Ca\(^{2+}\)]\(_{\text{ER}}\) was lower than in the Eos treatment alone but higher than with CPA alone. This suggests that the CPA-sensitive component is just partially responsible for the observed Eos-induced ER Ca\(^{2+}\) accumulation. After 60 min of incubation, the results were quite similar with CPA (Fig. 5B, gray bar), while the Eos effect on [Ca\(^{2+}\)]\(_{\text{ER}}\) increases was dramatically enhanced (Fig. 5B, white bar). In the latter condition, the presence of CPA was still able to reduce the [Ca\(^{2+}\)]\(_{\text{ER}}\) (Fig. 5B, striped bar). When the effect of inhibitors was tested on NES-YC3.6 seedlings, we observed that treatment with CPA for 10 min barely affected the [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. 5C, gray bar), while treatment with Eos led to an increase of [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Fig. 5C, gray bar) that was further slightly increased by the simultaneous addition of CPA (Fig. 5C, striped bar). Extension of the treatment to 60 min caused similar, but more pronounced, results.

In summary, the combined analyses of Ca\(^{2+}\) dynamics in ER and cytosol with Ca\(^{2+}\)-ATPase inhibitors revealed that, under basal conditions, CPA-sensitive ECAs substantially contribute to ER Ca\(^{2+}\) accumulation but have a minor role in the control of [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels. Application of Eos compromised the activity of the ACA pumps that are fundamental for the maintenance of basal [Ca\(^{2+}\)]\(_{\text{cyt}}\) in resting conditions but did not prevent the accumulation of Ca\(^{2+}\) in the ER, which
actually increased. This increase was reduced, but not fully suppressed, by CPA. Considering that under the applied conditions CPA suppressed ECA activity, these results indicate that, under elevated \([\text{Ca}^{2+}]_{\text{cyt}}\), ECAs contribute to \([\text{Ca}^{2+}]_{\text{ER}}\) accumulation, but other transport systems are also involved. We cannot exclude that the Eos concentration used in this study was sufficient to inhibit ACA localized at cellular membranes such as PM and tonoplast (Benza and De Michelis, 2011; Bose et al., 2011) but not fully effective in completely blocking the activity of the ER-resident ACAs (e.g. ACA2; Harper et al., 1998). Hence, it is plausible that the observed elevation of cytosolic \(\text{Ca}^{2+}\) removal, leads to higher ER \(\text{Ca}^{2+}\) accumulation.

**CONCLUSION**

In this work, we report (1) the generation of the CRT-D4ER Cameleon reporter protein as a suitable tool for the analysis of \(\text{Ca}^{2+}\) status and dynamics in the ER of plant cells; (2) the analysis of ER \(\text{Ca}^{2+}\) dynamics in root tip cells in response to defined stimuli; and (3) the evaluation of the contribution of ECA and ACA \(\text{Ca}^{2+}\)-ATPases to the control of ER \(\text{Ca}^{2+}\) homeostasis.
The application of CRT-D4ER enables the study of the dynamics of \([\text{Ca}^{2+}]_{\text{ER}}\) and its interconnection with cytosolic \(\text{Ca}^{2+}\) signatures in different cell types, genetic backgrounds, and developmental and stress-response processes.

Using this tool, we comparatively analyzed the dynamics of \([\text{Ca}^{2+}]_{\text{ER}}\) and \([\text{Ca}^{2+}]_{\text{cyt}}\) in response to different stimuli like ATP, 1-Glu, NaCl, and alternate applications of depolarizing and hyperpolarizing buffer. In conclusion, all our data do not support a significant role of \([\text{Ca}^{2+}]_{\text{ER}}\) as a source of \(\text{Ca}^{2+}\) release that contributes to the formation of cytosolic \(\text{Ca}^{2+}\) signatures, at least in the cell types and during the responses investigated in this study. Instead, our data support the hypothesis that, in plant cells, the ER functions primarily as a mimicking system for cytosolic \(\text{Ca}^{2+}\) signaling. These findings point to fundamental differences in the role of the ER for cellular \(\text{Ca}^{2+}\) dynamics in plants and animals.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (Arabidopsis thaliana) plants used in this study were of the Columbia ecotype. Plants were grown on 16/8-h cycles of light (70 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)) at 22°C and 75% relative humidity. Seeds of Arabidopsis were surface sterilized by vapor-phase sterilization (Clough and Bent, 1998) and plated on one-half Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 0.1% (w/v) Suc and 0.05% (w/v) MES, pH 6.0, and solidified with 0.8% (w/v) plant agar (Duchefa). After stratification at 4°C in the dark for 3 d, seeds were transferred to the growth chamber with 16/8-h cycles of light (70 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)) at 24°C. The plates were kept vertically. Seedlings used for the analyses were 7 to 8 d old, which corresponds to an average root length of 3 cm.

DNA Constructs

In order to generate the binary vectors for the expression of the Cameleon ER probes in plants, we inserted the single CaMV 335 promoter and the CaMV poly(A) terminator in the polylinker of the pGreen0029 binary vector (Hellens et al., 2000) by using the KpnI and SacI restriction sites, respectively. The CaMV 335 and CaMV poly(A) were PCR amplified using the 335-CaMV cassette vector as a template (http://www.pgren.uk.ac.uk/JJT/JJT.htm) and the following primer pairs: 335-For, 5′-CATGGtactGATATCGTACCCCTACTCCA-3′; 335-Rev, 5′-CATGggtaccGATATCGTACCCCTACTCCA-3′; YFd1ER, 5′-CATGggtaccGATATCGTACCCCTACTCCA-3′; D1-For, 5′-CATGGggtaccATCGATCTG-ACTGCCCTGAAATCACCAGT-3′; and D1-Rev, 5′-CATGggtaccATCGATCTG-ACTGCCCTGAAATCACCAGT-3′. The binary vectors were then introduced into the Agrobacterium tumefaciens GV3101 strain.

Transgenic Plants and Tobacco Transient Transformation

The A. tumefaciens strains obtained as reported above were used to generate transgenic Arabidopsis plants by the floral dip method (Clough and Bent, 1998). For each construct, 15 Arabidopsis independent transgenic lines were selected, and four independent lines were employed for imaging experiments. Experiments were carried out in seedlings of the T1 and T2 generations for CRT-D4ER transgenic lines. Mature T2 plants were affected by silencing. To minimize this problem, work is in progress to test different vector backbones and promoters.

Transient expression of CRT-D4ER in tobacco (Nicotiana benthamiana) epidermal leaf cells was performed as described by Waadt and Kudla (2008).

Seeding Preparation for \(\text{Ca}^{2+}\) Imaging

For root cell imaging, 7-d-old seedlings grown vertically were prepared accordingly to Behera and Kudla (2013) in dedicated chambers and overlaid with wet cotton in order to continuously perfuse the root with the imaging solution (5 mM KCl, 10 mM MES, 10 mM \(\text{Ca}^{2+}\), pH 5.8, adjusted with Tris for ATP and 1-Glu, or 0.1 mM KCl, 10 mM MES, 1 mM \(\text{Ca}^{2+}\), pH 5.8, adjusted with Tris for NaCl). The shoot was not submerged in the solution. 1-Glu and ATP were added as disodium or magnesium salt, respectively, to the chamber by perfusion with the same solution. For chemical treatments, seedlings were preincubated for 10 or 60 min in 5-cm petri dishes in the imaging solution supplemented with 25 \(\mu\text{M}\) CPA, 0.5 \(\mu\text{M}\) Eos, or a combination of both. Control seedlings were kept for the same times in the imaging solution supplemented with 0.25% (w/v) dimethyl sulfoxide. Seedlings were then transferred to the imaging chamber and allowed to recover for approximately 7 min prior to measurement.

Time-Lapse \(\text{Ca}^{2+}\) Imaging and Confocal Microscopy Analyses

Cameleon seedling roots and leaves were imaged in vivo by an inverted fluorescence Nikon microscope (Ti-E; http://www.nikon.com/) with a CFI plantfluor 4X numerical aperture 0.13 dry objective and a CFI PLAN APO 20×/0.70 dry objective. Excitation light was produced by a fluorescent lamp (Prior Lumen 200 PRO; Prior Scientific; http://www.prior.com) at 440 nm (436/20 nm) set to 20%. Images were collected with a Hamamatsu Dual CCD camera (ORCA-D2; http://www.hamamatsu.com/). For Cameleon analysis, the FRET CFP/YFP optical block A11400-03 (emission 1, 483/32 nm for CFP; emission 2, 542/27 nm for FRET) with a dichroic 510-nm mirror (Hamamatsu) was used for the simultaneous CFP and FRET acquisitions (citrine for D1, YFP for D4, and cpVenus for YC3.6). Exposure time was from 100 to 400 ms with a 2 × 2 CCD binning for cytosolic Cameleon (NIES-YC3.6) and a 4 × 4 CCD binning for ER Cameleons (CRT-D1ER and CRT-D4ER). Images where acquired every 5 s.

Filters and the dichroic mirror were purchased from Chroma Technology (www.chroma.com/). NIS-Elements (Nikon; http://www.nis-elements.com/) was used as a platform to control microscope, illuminator, camera, and post-acquisition analyses.

With regard to time-course experiments, fluorescence intensity was determined over regions of interest that correspond to the root tip zone. FRET and CFP emissions of the analyzed regions of interest were used for the ratio (R) calculation (FRET/CFP) and normalized to the initial ratio (R0) and plotted versus time (ΔR/Δt). The background was independently subtracted for both channels before calculating the ratio.

Confocal microscopy analyses were performed using Nikon C2 (http://www.nikoninstruments.com) and Leica SP5 (http://www.leica-microsystems.com) laser scanning confocal imaging systems. For Cameleon-dependent citrine (D1), YFP (D4), and cpVenus (YC3.6), excitation was at 488 nm and emission was between 525 and 540 nm. For mCherry detection, excitation was at 561 nm and emission was between 590 and 620 nm. Image analyses were done with the ImageJ bundle software (http://rsb.info.nih.gov/ij/).

For \(\text{Ca}^{2+}\) imaging analyses, roots were imaged with a 63×/1.20 NA oil objective (HCXPL PL APO, numerical aperture 1.20 water immersion), and the Cameleons were excited with the 488-nm line of the argon laser with 15% of total power. The CFP and FRET emissions were collected at 473 to 505 nm and 526 to 536 nm, respectively, and the pinhole diameter was 2 to 4 airy units depending on the line used. Images were collected every 5 s. The false color ratio images were obtained by using the NIS-Elements (Nikon; http://www.nis-elements.com/). For time-course experiments, fluorescence intensity was determined over regions of interest that correspond to single cells in the root tip zone. FRET and CFP emissions of the analyzed regions of interest were used for the ratio (R) calculation (FRET/CFP) and normalized to the initial ratio (R0) and plotted versus time (ΔR/Δt).

All the data are representative of at least six independent cells or roots analyzed unless otherwise stated. Reported traces are representative ones.
In Vivo Dynamic Range of CRT-D4ER

In order to determine the in vivo dynamic range of the CRT-D4ER probe ($\Delta R_{\text{max}}/R_0$), we performed experiments in root tip seedlings incubated with Ca$^{2+}$-ATPase inhibitors. The measured $R_\text{max}$ was $2.71 \pm 0.021$ in the presence of $25 \mu M$ CPA after 10 min of incubation, whereas the measured $R_\text{max}$ was $3.78 \pm 0.18$ in the presence of $0.5 \mu M$ Eos for 60 min. Hence, the calculated $\Delta R_{\text{max}}/R_0 = 0.395$ shows that the responses to the different observed stimuli did not lead to a probe saturation.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Subcellular distribution of CRT-DIER in transgenic Arabidopsis plants and simultaneous detection of CFP and FRET emissions.

Supplemental Figure S2. FRET detection in Arabidopsis seedlings expressing the CFP-DIER (ER Cameleon) probe challenged with 1 mM L-Glu. ATP analyzed by confocal laser scanning microscopy.

Supplemental Figure S3. [Ca$^{2+}$]$_{\text{cyt}}$ and [Ca$^{2+}$]$_{\text{ER}}$ dynamics in root cells of Arabidopsis seedlings expressing the NES-YC3.6 (cytosolic Cameleon) and CRT-DIER (ER Cameleon) probes challenged with 0.01 mM ATP.

Supplemental Figure S4. [Ca$^{2+}$]$_{\text{cyt}}$ and [Ca$^{2+}$]$_{\text{ER}}$ dynamics in root cells of Arabidopsis seedlings expressing the NES-YC3.6 (cytosolic Cameleon) and CRT-DIER (ER Cameleon) probes challenged with repetitive depolarizing-hyperpolarizing buffer changes in the presence or absence of EGTA.

Supplemental Text S1. Generation and analysis of Arabidopsis plants expressing the CRT-DIER Cameleon probe.

Supplemental Movie S1. Series of cytosolic Ca$^{2+}$ ratio images (low magnification, 4x) of an Arabidopsis seedling root tip expressing NES-YC3.6 challenged with 1 mM L-Glu.

Supplemental Movie S2. Series of cytosolic Ca$^{2+}$ ratio images of an Arabidopsis seedling root tip expressing NES-YC3.6 challenged with 1 mM L-Glu.

Supplemental Movie S3. Series of cytosolic Ca$^{2+}$ ratio images of an Arabidopsis seedling root tip expressing NES-YC3.6 challenged with 1 mM L-Glu acquired by confocal laser scanning microscopy.

Supplemental Movie S4. Series of ER Ca$^{2+}$ ratio images of an Arabidopsis seedling root tip expressing CRT-DIER challenged with 1 mM L-Glu acquired by confocal laser scanning microscopy.

Supplemental Movie S5. Series of ER Ca$^{2+}$ ratio images of an Arabidopsis seedling root tip expressing CRT-DIER challenged with 0.5 mM ATP analyzed by confocal laser scanning microscopy.

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