Comparative Analysis of MAMP-induced Calcium Influx in Arabidopsis Seedlings and Protoplasts

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Introduction

In eukaryotic cells, calcium (Ca2+) ions act as important second messengers that regulate many cellular processes. In plant cells, cytoplasmic Ca2+ concentrations ([Ca2+]cyt) are generally kept low, but certain abiotic (light, cold, heat, drought) and biotic (infection, elicitor treatment) stimuli can induce a rapid transient increase in [Ca2+]cyt by influx of Ca2+ from the extracellular environment or by release from internal stores (McAinsh and Pittman 2009). The recognition of microbe- or danger-/damage-associated molecular patterns (MAMPs or DAMPs, respectively) by specific pattern recognition receptors (PRRs), is known to trigger characteristic Ca2+ signatures (Lecourieux et al. 2006, Aslam et al. 2009). PRRs represent crucial components of plant innate immunity. Typically, they are receptor-like kinases (RLKs) anchored in the plasma membrane and comprise an N-terminal extracellular domain for ligand binding, a transmembrane domain, and a C-terminal cytoplasmic kinase domain for intracellular signaling. Several PRRs of the dicotyledonous model plant Arabidopsis thaliana have been characterized in detail, including FLAGELLIN SENSING 2 (FLS2) and ELONGATION FACTOR-THERMO UNSTABLE RECEPTOR (EFR), which activate downstream signaling upon detection of peptide epitopes of bacterial flagellin (flg22) and EF-Tu (elf18), respectively (Gómez-Gómez and Boller 2000, Zipfel et al. 2004). Similarly, chitin oligomers originating from fungal cell walls are recognized by direct binding to the PRR CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Miya et al. 2007, Liu et al. 2012, Petutschnig et al. 2010), whereas the receptor for lipopolysaccharides (LPS), originating from the outer membrane of Gram-negative bacteria, has not yet been identified (Newman et al. 2013). In Arabidopsis the DAMPs Pep1 through Pep6 serve as endogenous elicitors, which are short peptides stimulating innate immune responses (Huffaker et al. 2006). The PRR PEPTIDE RECEPTOR 1 (PEPR1) functions as receptor for Pep1-Pep6, whereas PEPR2 acts as a receptor for Pep1 and Pep2 (Yamaguchi et al. 2010). The RLK BRASSINOSTEROID INSENSITIVE 1–ASSOCIATED RECEPTOR KINASE 1 (BAK1) does not directly bind MAMPs/DAMPs, but serves as a co-receptor for FLS2, EFR, PEPR1/PEPR2 and possibly other...
PRRs (Kim et al. 2013). A set of canonical cellular responses is typically initiated by activation of PRRs, including extracellular alkalization (Felix et al. 1999), rapid transient increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) (Aslam et al. 2009, Jaworzutki et al. 2010), activation of \([\text{Ca}^{2+}]_{\text{cyt}}\)-dependent protein kinases (CDPKs; Boudsocq et al. 2010), generation of reactive oxygen species (ROS, Felix et al. 1999), alterations in lateral plasma membrane organization (Keinath et al. 2010), initiation of mitogen-activated protein kinase (MAPKK/KKK) cascades (Asai et al. 2002), and transcriptional activation of a set of defense-related genes (Zipfel et al. 2004). Although a range of candidate \([\text{Ca}^{2+}]_{\text{cyt}}\) channels in plants is known (Wheeler and Brownlee 2008), the molecular identity of the channels mediating the MAMP/DAMP-induced cellular \([\text{Ca}^{2+}]_{\text{cyt}}\) influx in plants remains elusive. Previous pharmacological studies suggest a significant contribution of ionotropic glutamate receptor (iGluR)-like channels to this process (Kwiatkaal et al. 2011, Vatsa et al. 2011a, Kwiatkaal et al. 2012); however, genetic evidence in support of this possibility has only recently emerged (Li et al. 2013, Manzoor et al. 2013).

There exist a number of methods to measure steady-state levels and dynamics of \([\text{Ca}^{2+}]_{\text{cyt}}\) in plants. One commonly used procedure employs the genetically encoded proteinaceous bioluminescent \([\text{Ca}^{2+}]_{\text{cyt}}\) sensor apoaequorin from the jellyfish \textit{Aequorea victoria}. The complex formed between apoaequorin and its prosthetic group, the luciferin coelenterazine, is termed aequorin, which upon binding of three \([\text{Ca}^{2+}]_{\text{cyt}}\) ions emits light at a wavelength of 470 nm. The emitted light can be quantified as bioluminescence \emph{via} photomultiplier tubes or charge-coupled devices (Knight et al. 1991). Using microplate readers for bioluminescence quantification, \([\text{Ca}^{2+}]_{\text{cyt}}\) measurements on the basis of aequorin are suitable for medium- to high-throughput screening (Ranf et al. 2012). By decreasing sample size and increasing the rate of data collection in individual samples, it is possible to obtain high-resolution kinetics with good reproducibility. Such measurements can be useful to discover even small phenotypic changes in mutant lines or pharmacologically treated samples.

A marked disadvantage of the bioluminescent aequorin \([\text{Ca}^{2+}]_{\text{cyt}}\) reporter system and other genetically encoded \([\text{Ca}^{2+}]_{\text{cyt}}\) sensors is the need for integration of the respective biosensor constructs into suitable plant lines by either transformation or crossing, which are both laborious and time-consuming. An attractive alternative to stable integration of the reporter systems into plant genomes is transient expression of the respective constructs. Various transient expression systems have been established for plants, including particle bombardment (Panstruga 2004), \textit{Agrobacterium tumefaciens}-mediated expression (Gelvin 2003) and protoplast transfection (Davey et al. 2005, Yoo et al. 2007). Especially the latter method has been widely used and seminal results have been obtained on the basis of \textit{Arabidopsis} leaf mesophyll protoplasts. This includes, for example, the elucidation of MAMP-triggered MAPK cascades (Asai et al. 2002) and the functional analysis of defense signaling \emph{via} \([\text{Ca}^{2+}]_{\text{cyt}}\)-dependent protein kinases (Boudsocq et al. 2010).

Protoplast- and cell culture-based \([\text{Ca}^{2+}]_{\text{cyt}}\) measurements using the aequorin reporter have been performed in \textit{Arabidopsis} (Demidchik et al. 2004, Carpaneto et al. 2007, Jaworzutki et al. 2010), but also in other plant systems such as tobacco (Haley et al. 1995, Mazars et al. 1997, Lecourieux et al. 2005), parsley (Blume et al. 2000), wheat, barley and carrot (Gilroy et al. 1989). Typically, \([\text{Ca}^{2+}]_{\text{cyt}}\) quantification was combined with a variety of abiotic and biotic stress stimuli. However, the validity of this approach has so far not been proven by systematic direct comparison of the protoplast-derived responses with those obtained with the corresponding intact plant systems; in particular, there have been no previous reports on MAMP-/DAMP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) signatures in \textit{Arabidopsis} protoplasts.

Here we report on \([\text{Ca}^{2+}]_{\text{cyt}}\) measurements on the basis of transient expression of the \([\text{Ca}^{2+}]_{\text{cyt}}\) biosensor aequorin in \textit{Arabidopsis thaliana} leaf mesophyll protoplasts. We systematically compared MAMP- and DAMP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) signatures recorded in the protoplast system with those obtained in seedlings of stable transgenic plant lines, both in the context of pharmacological inhibitor and genetic complementation experiments. Our data demonstrate that MAMP-/DAMP-triggered \([\text{Ca}^{2+}]_{\text{cyt}}\) transients in leaf mesophyll protoplasts are overall comparable to the responses seen in intact seedlings. We conclude that the leaf protoplast system is suitable for pharmacological and genetic analysis of MAMP-/DAMP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) signatures. Our study thus provides a solid basis for the future application of protoplast-based measurements of \([\text{Ca}^{2+}]_{\text{cyt}}\) signatures using the aequorin sensor in \textit{Arabidopsis} as well as in other plant species.

**Results**

\textit{Arabidopsis} leaf mesophyll protoplasts are responsive to various elicitors

To find out whether the \([\text{Ca}^{2+}]_{\text{cyt}}\) signature in leaf mesophyll protoplasts reflects the \([\text{Ca}^{2+}]_{\text{cyt}}\) response seen in intact plant seedlings, we first isolated protoplasts from rosette leaves of the transgenic \textit{Arabidopsis} line, Col-0 [pMAQ2], which expresses the apoprotein of the \([\text{Ca}^{2+}]_{\text{cyt}}\) sensor aequorin in the plant cytoplasm (Knight et al. 1991). To compensate for the lack of the apoplastic \([\text{Ca}^{2+}]_{\text{cyt}}\) store present in intact plant tissues, we incubated protoplasts in buffer containing 2 mM \([\text{Ca}^{2+}]_{\text{cyt}}\), which is within the \([\text{Ca}^{2+}]_{\text{cyt}}\) range typically found in the plant extracellular space (10 \(\mu\text{M}\) to 10 mM; Bush 1995, Stael et al. 2012). Subsequently, the leaf mesophyll protoplasts were challenged with different MAMPs and DAMPs and changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) were recorded over time. Results were compared to data obtained in parallel experiments employing intact seedlings of the transgenic Col-0 [pMAQ2] line grown in hydroponic culture.

The kinetic properties of the flg22-induced cytoplasmic \([\text{Ca}^{2+}]_{\text{cyt}}\) transients observed in mesophyll protoplasts were comparable to the ones monitored with \textit{Arabidopsis} seedlings, with minor differences. In both systems treatment with flg22 caused a rapid transient increase in \([\text{Ca}^{2+}]_{\text{cyt}}\). The \([\text{Ca}^{2+}]_{\text{cyt}}\) spike, which was nearly identical in amplitude in both systems (\(\Delta[\text{Ca}^{2+}]_{\text{cyt}}\) ca. 120–130 mM; Figs. 1a and 2a; note that absolute values are
subject to experimental variation), was followed by a slow and gradual decrease during the recovery phase (Fig. 1a). While in intact seedlings the \([Ca^{2+}]_{\text{cyt}}\) started to increase at about 40 s after administration of flg22 and typically reached a maximum at 60 to 120 s, the response of protoplasts was slightly delayed. In protoplasts, \(Ca^{2+}\) influx started at ca. 60 s and maximum \([Ca^{2+}]_{\text{cyt}}\) was reached at about 120 to 180 s (Figs. 1 and 2a). The kinetics of the recovery phase was similar in seedlings and protoplasts; in both systems \([Ca^{2+}]_{\text{cyt}}\) declined back to resting levels at ca. 25 min (1500 s) after treatment with flg22 (Fig. 1a).

In seedlings, \([Ca^{2+}]_{\text{cyt}}\) dropped rapidly and then slowly phased out, typically resulting in a concavely shaped recovery curve (Kwaaitaal et al. 2011, Ranf et al. 2011, Ranf et al. 2012), whereas in protoplasts the decrease in \([Ca^{2+}]_{\text{cyt}}\) was less steep and steadier (Fig. 1a).

A characteristic feature of the experimentally determined \(Ca^{2+}\) signature is the so-called ‘injection peak’, which is presumably caused by physical perturbation of the system upon injection of the MAMP solution into microplate wells harboring the apoaequorin-expressing specimen. Injection may also cause the generation of ion microgradients, osmotic effects or temperature changes, which collectively may be responsible for this frequently observed and variable response (Kwaaitaal et al. 2011, Ranf et al. 2011). In both protoplasts and seedlings, this peak occurred instantly after MAMP injection, indicating that both systems are equally responsive to such perturbations (Fig. 1b; note that the amplitude of the injection peak is variable and subject to experimental variation). Resting levels of \([Ca^{2+}]_{\text{cyt}}\) prior to MAMP application were similar (\([Ca^{2+}]_{\text{cyt}}\) ca. 70 nM) in seedlings and protoplasts (Fig. 1b) and comparable to previously reported values in Arabidopsis seedlings that were also quantified by apoaequorin-based measurements (Aslam et al. 2009, Leworutzki et al. 2010, Ranf et al. 2011, Kwaaitaal et al. 2012), whereas somewhat higher \([Ca^{2+}]_{\text{cyt}}\) were determined in plant protoplasts using \(Ca^{2+}\) indicator dyes (Gilroy et al. 1986, Gilroy et al. 1989).

We next assessed a range of additional MAMPs and DAMPs as triggers of \([Ca^{2+}]_{\text{cyt}}\) signatures. These included, in addition to flg22, the bacterial peptide MAMP elf18, the plant peptide DAMP Pep1, the fungal cell wall carbohydrate MAMP, chitin, and the bacterial cell wall MAMP, LPS. Previous measurements have shown the responsiveness of seedlings grown in hydroponic culture to these elicitors (Gust et al. 2007, Kwaaitaal et al. 2011, Kwaaitaal et al. 2012, Ranf et al. 2011, Ranf et al. 2012). In protoplasts, each of these MAMPs/DAMPs caused a rapid and transient increase in \([Ca^{2+}]_{\text{cyt}}\) (Fig. 2). The elf18-induced cytoplasmic \(Ca^{2+}\) transient in protoplasts was of similar amplitude as in seedlings (\(\Delta[Ca^{2+}]_{\text{cyt}}\) ca. 80 nM), yet was delayed and showed a slower increase of the \(Ca^{2+}\) influx (Fig. 2b). By contrast, the \(Ca^{2+}\) signatures induced by Pep1 (Fig. 2c) or chitin (Fig. 2d) occurred at the same rate in protoplasts and seedlings, but the amplitudes were significantly higher in protoplasts when compared to seedlings (130–190 nM versus 70–80 nM), and the decline during the recovery phase happened earlier and proceeded faster (Fig. 2c and d). Finally, bacterial LPS induced a rapid \(Ca^{2+}\) transient only in protoplasts, but not in seedlings, where LPS caused no noticeable increase in \([Ca^{2+}]_{\text{cyt}}\) during the time of examination (Fig. 2e), comparable to the injection of solvent (water).

In summary, we found that the Arabidopsis protoplast system is responsive to all tested MAMPs/DAMPs, including flg22, elf18, Pep1, chitin, and LPS, but show \([Ca^{2+}]_{\text{cyt}}\) kinetics with some distinctive features regarding timing, amplitude and recovery phases compared to intact seedlings.

**Pharmacological inhibition of flg22-induced \([Ca^{2+}]_{\text{cyt}}\) transients in Arabidopsis mesophyll protoplasts**

Pharmacological inhibition is a common and often straightforward experimental approach to interfere with biological processes. It may provide meaningful insights into the underlying signaling pathways and can be applied at small scale (individual substances) or in high-throughput screens (compound libraries), an experimental approach known as chemical genetics (McCourt and Desveaux 2010). To find out whether aequorin-based \(Ca^{2+}\) measurements in leaf mesophyll protoplast are suitable for and react in a similar fashion to...
pharmacological interference, we pretreated transgenic Col–0 [pMAQ2] seedlings and protoplasts with various concentrations of the non-selective Ca$^{2+}$ channel blocker, lanthanum chloride (LaCl$_3$), or the pan-specific kinase inhibitor, staurosporine. Both LaCl$_3$ and staurosporine have previously been shown to effectively block cytoplasmic Ca$^{2+}$ signatures in various experimental contexts and plant systems (Knight et al. 1997, Mazars et al. 1997, Lecourieux et al. 2002, Rentel and Knight 2004, Lecourieux et al. 2005, Vadassery et al. 2009, Kurusu et al. 2011, Kwaaitaal et al. 2011, Vatsa et al. 2011b). In protoplasts, 1 mM LaCl$_3$ or 5 μM staurosporine completely inhibited the flg22-triggered Ca$^{2+}$ transient, whereas at lower concentrations (50 and 100 μM LaCl$_3$, 100 and 500 nM staurosporine) the response was only partially compromised, as revealed by a delayed Ca$^{2+}$ spike and/or a reduced peak height (Fig. 3a and b). We extended these measurements and determined half-maximal inhibitory concentrations (IC$_{50}$ values) for protoplasts and seedlings. For both compounds typical sigmoidal dose-response curves were obtained (Fig. 3c–f), from which IC$_{50}$ values of 80 μM for LaCl$_3$ and 251 nM for staurosporine were derived for the protoplast system. For seedlings, the corresponding IC$_{50}$ values were 76 μM for LaCl$_3$ (similar to protoplasts) and 1080 nM for staurosporine (ca. four-fold higher than in protoplasts). In sum, these experiments
demonstrate the suitability of the leaf mesophyll protoplast system for chemical inhibitor studies in the context of MAMP/DAMP-induced Ca$^{2+}$ transients. Generally, similar inhibitor kinetics might be expected in protoplast-based experiments compared to intact seedlings, with the tendency of protoplasts being more sensitive to lower inhibitor concentrations.

Transient expression of the Ca$^{2+}$ reporter apoaequorin in Arabidopsis wild-type protoplasts

Next we explored whether transient expression of the Ca$^{2+}$ sensor is a suitable strategy to monitor Ca$^{2+}$ signatures. Therefore, leaf mesophyll protoplasts derived from Col-0 wild type plants were transfected with a plasmid encoding an N-terminally fluorophore (mCherry)-labeled version of the cytoplasmic Ca$^{2+}$ reporter, apoaequorin (mCherry-AEQ). At 12–16 hours post transfection, we detected the specific red fluorescent signal indicating mCherry-AEQ accumulation in the cytoplasm and possibly the nucleus of transfected protoplasts (Fig. 4a). When such transfected protoplasts (Col-0 [mCherry-AEQ]) were treated with flg22, the recorded Ca$^{2+}$ transients were comparable to Ca$^{2+}$ signatures obtained with protoplasts from the transgenic Arabidopsis line Col-0 [pMAQ2], although we monitored a slightly lower amplitude (90–100 nM versus 120–130 nM; Fig. 4c). Without MAMP elicitation, protoplasts showed no increase in [Ca$^{2+}$]$_{cyt}$ over time, demonstrating that the Ca$^{2+}$ signal in the transfected protoplasts is stimulus-dependent (Fig. 4c). These results
show that the MAMP-induced Ca^{2+} response of mCherry-AEQ-transfected mesophyll protoplasts qualitatively and quantitatively resembles the response of protoplasts isolated from the transgenic Col–0 \([\text{pMAQ2}}] \) line. The data further indicate that the translational fusion of apoaequorin with the mCherry fluorophore does not affect the activity of the Ca^{2+} sensor.

We also tested a modified version of the mCherry-AEQ reporter construct, which harbors an additional C-terminal nuclear localization signal (NLS) thereby directing the polypeptide to the nucleus (mCherry-AEQ-NLS). The respective fusion protein accumulated in the nuclei of transfected protoplasts with no or only little cytoplasmic localization (Fig. 4b). Again, flg22-triggered Ca^{2+} signatures recorded with transfected protoplasts expressing this apoaequorin variant with nuclear localization signal were similar in shape as the response curve obtained with the cytoplasmic apoaequorin version, although the amplitude was somewhat lower under identical experimental conditions \((\Delta [Ca^{2+}]_{\text{cyt}} \text{ ca. } 70 \text{ nM})\) than the transients recorded with the cytoplasmic apoaequorin version \((\Delta [Ca^{2+}]_{\text{cyt}} \text{ ca. } 120 \text{ nM}; \text{compare Fig. 4c and d})\). Also, the onset of Ca^{2+} influx in the nucleus was delayed by ca. 30 seconds in comparison to the cytoplasmic recordings. This finding is consistent with similar comparative measurements of flg22-induced Ca^{2+} transients in the cytoplasm and nucleus of cultured tobacco cells, although the delay of the nuclear signal in the tobacco system was longer, amounting to a difference in timing of the Ca^{2+} transients of up to 15 min (Lecourieux et al. 2005).

Based on results with pharmacological inhibitors the authors of the latter study argued that the rise in nuclear \([Ca^{2+}]_{\text{nuc}}\) is unlikely to result from merely the passive diffusion of Ca^{2+} from the cytosol, but may rather originate from an influx from internal stores such as the intermembrane space of the nuclear envelope or the ER-nucleus membrane continuum (Lecourieux et al. 2005).

**Transient apoaequorin expression in bak1-4 mutant protoplasts**

Results from a previous report revealed that the increase of \([Ca^{2+}]_{\text{cyt}}\) in response to the peptide MAMPs flg22 and elf18, but not in response to the fungal carbohydrate MAMP, chitin, was delayed and reduced in bak1-3 and bak1-4 mutants (Ranf et al. 2011). BAK1 acts as a co-receptor of the flg22 receptor, FLS2, and the elf18 receptor, EF-Tu, but not of the chitin

![Fig. 4 Continued](https://academic.oup.com/pcp/article-abstract/55/10/1813/2756066/Comparative-Analysis-of-MAMP-induced-Calcium/fig4-continued)

### Fig. 4

**Fig. 4** Transient expression of fluorophore-tagged apoaequorin variants in wild-type *Arabidopsis* leaf mesophyll protoplasts. (a) Transient expression of N-terminally mCherry-tagged apoaequorin (mCherry-AEQ). Left: fluorescence signal; right: fluorescence signal and bright field overlay; bottom: schematic representation of the expression construct (35S, cauliflower mosaic virus 35S promoter). Bar, 10 \(\mu\)m. (b) Transient expression of N-terminally mCherry-tagged apoaequorin harboring a C-terminal nuclear localization signal (mCherry-AEQ-NLS). Left: fluorescence signal; right: fluorescence signal and bright field overlay; bottom: schematic representation of the expression construct (35S, cauliflower mosaic virus 35S promoter; NLS, nuclear localization signal). Bar: 10 \(\mu\)m. (c, d) *Arabidopsis* leaf mesophyll protoplasts were either isolated from the transgenic apoaequorin-expressing Col-0 \([\text{pMAQ2}}] \) line or Col-0 wild type plants. Col-0-derived protoplasts were either transfected with mCherry-AEQ (c) or mCherry-AEQ-NLS (d). Non-transfected Col-0 \([\text{pMAQ2}}] \)-derived protoplasts and transfected Col-0-derived protoplasts were either treated with 1 \(\mu\)M flg22 or \(\text{Wc}_2\) buffer at the start of the measurements. Curves are from representative experiments each showing the mean (± SEM) of three biological replicates and each based on at least three technical replicates, respectively. Note that the black curves (Col-0 \([\text{pMAQ2}}] \)) in (c) and (d) represent the same data and are identical.
receptor, CERK1 (Gimenez-Ibanez et al. 2009). To unravel whether the differential response seen in seedlings of the bak1 mutant background can be reproduced in the mesophyll protoplast system, we compared MAMP-induced Ca\(^{2+}\) signatures in (1) protoplasts derived from a transgenic aequorin-expressing wild-type line (Col-0 [pMAQ2]), (2) protoplasts isolated from a transgenic aequorin-expressing bak1-4 mutant line (bak1-4 [pMAQ2]; Ranf et al. 2011) and (3) protoplasts derived from the bak1-4 mutant transfected with the apoaequorin-encoding plasmid pMAQ2 (bak1-4 [pMAQ2]). In line with the previous findings (Ranf et al. 2011), the transgenic bak1-4 [pMAQ2] mutant line showed a delayed and somewhat reduced \([Ca^{2+}]_{c}\) signature in response to flg22 (Fig. 5a) and elf18 (Fig. 5b), but not in response to chitin (Fig. 5c), when compared to protoplasts derived from aequorin-expressing wild type plants. Importantly, the differential behavior in the bak1 mutant was faithfully phenocopied by mCherry-AEQ-transfected bak1-4 mesophyll protoplasts (Fig. 5). These data indicate that transient aequorin expression in leaf mesophyll protoplasts can be successfully combined with genetic pathway analysis using Arabidopsis mutants for protoplast isolation and transfection.

**fls2 mutant complementation by transient gene expression in leaf mesophyll protoplasts**

To further explore the potential of genetic pathway analysis in the context of MAMP-triggered Ca\(^{2+}\) signatures, we speculated that loss-of-gene functions could be restored by transient gene expression in protoplasts. For proof of concept we took advantage of an Arabidopsis fls2 mutant, in which expression of the pattern recognition receptor FLS2 is completely abolished by a T-DNA insertion (Gómez-Gómez and Boller 2000, Zipfel et al. 2004). Consequently, the fls2 mutant is insensitive to flg22 but retains responsiveness to other MAMPs. Protoplasts derived from a transgenic aequorin-expressing fls2 T-DNA mutant line (fls2 [pMAQ2]) were transfected with a plasmid encoding an epitope-tagged FLS2 version under the control of its native promoter (pCAMBIA-FLS2::FLS2-3xmyc-GFP) and treated with either flg22 or elf18. Non-transfected protoplasts of the aequorin-expressing fls2 mutant were unresponsive to flg22 (Fig. 6a) but retained responsiveness to elf18 (Fig. 6b), whereas the FLS2-transfected protoplasts (fls2 [pMAQ2] [FLS2p:FLS-3xmyc-GFP]) exhibited a flg22-triggered Ca\(^{2+}\) signature that was similar to the flg22-induced Ca\(^{2+}\) response in protoplasts derived from the aequorin-expressing wild-type line (Fig. 6a). The slightly different kinetics of the \([Ca^{2+}]_{c}\) response obtained in the transfected protoplasts might result from different receptor quantities in non-transfected and transfected protoplasts. As expected, the Ca\(^{2+}\) response to elf18 was indistinguishable in the different types of protoplasts used in this set of experiments (Fig. 6b). In conclusion, our data demonstrate that genetic complementation analysis is feasible by combining transient gene expression in mutant protoplasts with luminescence-based measurements of Ca\(^{2+}\) signatures.

**Discussion**

The results presented in this study demonstrate that Arabidopsis mesophyll protoplasts represent a versatile and convenient tool to determine MAMP-/DAMP-induced Ca\(^{2+}\) signatures in intact plant cells. Aequorin-expressing protoplasts largely reflect the \([Ca^{2+}]_{c}\) increases in response to...
some distinctive features regarding timing, amplitude and recovery phase of
Ca$^{2+}$ patterns. The faster responsiveness of the seedlings, reaching an earlier
maximum of [Ca$^{2+}$]$_{cyt}$ upon elicitation with flg22 and elf18, was
unexpected. Intuitively, one might predict a quicker response in
the protoplast system because the applied elicitors (MAMPs/ DAMPs) should rapidly diffuse and bind to their exposed cognate plasma membrane-resident receptors of the free-floating, spherical mesophyll protoplasts, which lack at least part of their cell walls. However, epidermal cells of intact seedlings may in-
trinsically react faster to MAMPs/DAMPs than leaf mesophyll
cells. Alternatively, lower levels of PRRs in mesophyll compared to
epidermal cells may cause a delayed response. Finally, extracellular Ca$^{2+}$ reservoirs differ in the seedling and protoplast system; while Ca$^{2+}$ may be present at high local concentrations in the apoplastic space of seedlings (possibly varying between 10 μM and 10 mM; Stael et al. 2012), it is provided as homogenous solution in the context of protoplast-based experi-
ments. However, the response of protoplasts was strongly reduced but not completely abolished when Ca$^{2+}$ was omitted from the medium, suggesting that MAMP/DAMP-triggered Ca$^{2+}$ influx remains operative at a wide range of concen-
trations. The most extreme difference between protoplasts and
seedlings in response to treatment with different elicitors was
observed with LPS. In contrast to protoplasts, seedlings showed no Ca$^{2+}$ transient upon LPS treatment (Fig. 2). Although we
used the same batch of LPS throughout our experiments, it
remains open whether the lack of a Ca$^{2+}$ response in seedlings
truly reflects inherent differences of both systems or whether it relates to different properties of commercial LPS batches/
preparations that have previously been reported (S. Ranf, personal communication, Schneider et al. 1997).

An obvious advantage of the protoplast system in compari-
sion to intact seedlings is the reduced complexity of cell types. While many different cell types may contribute to the Ca$^{2+}$ signature seen in seedlings or detached leaves, another experimental system used to conduct aequorin measurements (Grant et al. 2000, Aslam et al. 2009, Qi et al. 2010), leaf mesophyll protoplasts represent a homogenous and adjustable (with regard to the number of cells) type of experimental material. This feature is expected to minimize experimental variation and to confer reproducible data. Isolation of protoplasts usually yields an ample amount of experimental material. Consequently, the homogenous nature of the cell suspension allows direct comparison of different cellular responses upon treatment with different stimuli (e.g. elicitors, hormones, chemicals) using appropriate aliquots. Alternatively, different analytical procedures may be applied to a single batch of protoplasts following treatment with a specific stimulus. The results of such analyses based on homogenous cell/protoplast popu-
lations are expected to provide better comparability and repro-
ducibility in comparison to experiments carried out with whole
organisms (seedlings). Previous work has shown that also whole
seedlings, or shoots and roots separated therefrom, can be used

Fig. 6 Genetic complementation by transient gene expression in
Arabidopsis leaf mesophyll protoplasts. Arabidopsis leaf
mesophyll protoplasts isolated from the transgenic aequorin-
expressing Col-0 [pMAQ2] line (black curves), an aequorin-
expressing line with fls2 mutant background (fls2 [pMAQ2]; blue
curves), and an aequorin-expressing line with fls2 mutant
background transfected with pCAMBIA-FLS2p::FLS2-3xmyc-GFP
[fls2 [pMAQ2]] [LS2p-FLS2-3xmyc-GFP]; red curves) were treated with either
1 μM flg22 (a) or 1 μM elf18 (b) at the start of the measurements.
Curves are from representative experiments each showing the mean
(± SEM) of two biological replicates, all of which are based on at least
three technical replicates, respectively.

various elicitors seen in whole seedlings, which are typically
used to study Ca$^{2+}$ responses in Arabidopsis, but also revealed
some distinctive features regarding timing, amplitude and
recovery phase (Figs. 1 and 2; Kwaaitaal et al. 2011, Ranf et al.
2011, Kwaaitaal et al. 2012, Ranf et al. 2012). These differences are
unlikely a consequence of the different age of seedlings
(2 weeks) and leaves (5 to 6 weeks) used for protoplast
preparation since we obtained similar results with protoplasts
isolated from mature leaves and protoplasts isolated from
seedlings (Supplementary Fig. 1). The kinetic differences
observed in response to the applied stimuli may thus rather
result from the combination of different cell and tissue types in
the case of seedlings vs. a single cell type (mesophyll cells)
predominating the protoplast preparation. However, roots
have previously been shown to provide only a minor contribu-
tion to the flg22- and elf18-induced Ca$^{2+}$ signature and a sub-
standard contribution to the chitin- and Pep1-induced Ca$^{2+}$
signature in seedlings (Ranf et al. 2011). Thus, leaf cells including
mesophyll cells likely contribute a major proportion to the
seedling response. Alternatively or in addition, responses may
not be cell-autonomous in seedlings but rather involve feed-
back control between neighboring cells within the tissue,
thereby possibly giving rise to more complex Ca$^{2+}$ patterns.

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for protoplast preparation (Zhai et al. 2009, Bargmann and Birnbaum 2010). In combination with subsequent fluorescence-activated cell sorting (FACS) of plant protoplasts, a direct link between the tissue origin and shape of the Ca\(^{2+}\) signatures and consequent downstream responses could be achieved. Cell-type-specific expression of the Ca\(^{2+}\) reporter aequorin is already available by GAL4 transactivation of the aequorin gene in enhancer trap lines of Arabidopsis (Marti et al. 2013). Comparative analysis of the [Ca\(^{2+}\)]\(_{oc}\) elevation of these lines at seedling stage and as protoplasts could assist in deciphering the composition and origin of the so far observed [Ca\(^{2+}\)]\(_{oc}\) elevations obtained from the transgenic Col-0 [pMAQ2] line, which constitutively expresses aequorin without tissue-specificity. These new technical possibilities have the potential to make a marked contribution to the dissection of plant Ca\(^{2+}\) signaling pathways, including Ca\(^{2+}\) responses triggered by MAMPs/DAMPs.

A particular concern of using protoplasts for MAMP-induced Ca\(^{2+}\) measurements might be the liberation of plant cell wall fragments during protoplast preparation and/or the release of cellular content and debris upon undesired lysis of protoplasts during experiments. Both cell wall fragments and cellular debris could potentially act as DAMPs and thereby have an impact on the MAMP-triggered Ca\(^{2+}\) responses by either stimulating (priming) or desensitizing the protoplasts for the subsequent MAMP stimulus. The latter could be an alternative explanation for the delayed flg22- or elf-18-induced response of protoplasts (Figs. 1 and 2a, b). However, treatment with the endogenous elicitors Pep1 or the fungal MAMP chitin did not result in delayed Ca\(^{2+}\) responses of protoplasts in comparison to seedlings grown in hydroponic culture (Fig. 2b and c).

The protoplasts were prepared by a non-sterile isolation procedure; thus microbial contamination was unavoidable. Indeed, longer storage (e.g. overnight) of protoplasts at room temperature often resulted in a marked decline of the recorded Ca\(^{2+}\) flux, which we associated with enhanced bacterial growth in the protoplast-containing medium (Supplementary Fig. 2). By contrast, upon overnight storage at 4°C protoplasts retained their responsiveness to MAMP treatment and produced reliable and reproducible Ca\(^{2+}\) traces (Supplementary Fig. 2). Thus, protoplasts apparently remain viable and retain their capacity for generating Ca\(^{2+}\) transients for an extended time period, provided that bacterial contamination and growth is minimized, which enables their application in large-scale and time-consuming experiments.

Protoplasts can also be used for pharmacological inhibitor studies (Fig. 3) and genetic pathway analysis (Figs. 4–6). In fact, the protoplast system may be advantageous for inhibitor studies since some chemicals might be taken up more easily than by intact seedlings owing to the greater accessible surface area and the at least partially lacking cell walls. We conducted a comparative analysis in protoplasts and seedlings with two well-characterized inhibitors of Ca\(^{2+}\) mediated responses, the Ca\(^{2+}\) channel blocker LaCl\(_3\) and the kinase inhibitor staurosporine (Fig. 3). We determined a similar IC\(_{50}\) value for LaCl\(_3\) in both systems, which is not unexpected, since this Ca\(^{2+}\) blocker acts on the extracellular face of plasma membrane-localized Ca\(^{2+}\) channels. La\(^{3+}\) ions bind to the pore of Ca\(^{2+}\) channels, resulting in pore occlusion (Doering and Zamponi 2003). This process is dependent on the physical interaction between the La\(^{3+}\) ion and the channel pore, which should be independent of the biological system under examination. By contrast, staurosporine as pan-specific protein kinase inhibitor (Karaman et al. 2008) blocks a wide range of ATP-dependent mechanisms. Therefore it may interfere with diverse ATP-dependent and/or kinase-mediated processes, including to defense-associated signaling processes MAMP-induced cytoplasmic receptor kinase activities. In contrast to LaCl\(_3\), staurosporine has to enter cells to exert its effect. This process may take longer or may be less effective in cells of the tissue interior. In addition, unspecific binding to other targets or cellular components such as the cell wall may reduce the effective intracellular concentration. In any case, the apparent IC\(_{50}\) value for staurosporine was about four times higher in seedlings than in protoplasts (1080 nM vs. 251 nM, respectively).

For the analysis of genetic pathways, the presented system offers the possibility to transiently express the aequorin Ca\(^{2+}\) reporter in different genetic backgrounds (Figs. 4–6). This option renders mutant analysis faster and much more effective, since it alleviates researchers from the otherwise necessary introgression of transgenic aequorin reporter constructs into suitable plant lines by either transformation or crossing. Available single or higher order mutants can directly be used for protoplast isolation in combination with transfection, saving considerable time in comparison to conventional modes of analysis. For example, recent studies uncovered cyclic nucleotide-gated channels (Clough et al. 2000, Ali et al. 2007, Ma et al. 2009) and ionotropic glutamate receptor (iGluR)-like channels (Kwaitaal et al. 2011, Vatsa et al. 2011a, Kwaitaal et al. 2012, Manzoor et al. 2013, Li et al. 2013) as candidates that could mediate Ca\(^{2+}\) influx during plant-microbe interactions. Given that these proteins are encoded by medium-sized gene families, comprising 20 members each in Arabidopsis, conventional genetic analysis via introgression of the reporter construct into each of the different mutants is tedious and time-consuming. Besides for the identification of the actual channels, the procedure can also be used to study the role of presumed Ca\(^{2+}\) signal transduction components. It offers further the possibility to verify the presumed effect of mutants by complementation analysis (Fig. 6), saving the need for additional independent mutant alleles to prove the contribution of a particular gene in Ca\(^{2+}\) signaling. Co-transfection of multiple plasmids enables co-expression of aequorin reporter constructs together with gene complementation constructs within the same cell, further extending the options of mutant analysis. Thus, candidate genes can be tested and identified more quickly using the protoplast system and eventually verified by stable transgenic lines in a more targeted approach.

An additional aspect of the protoplast system is the possibility to deploy flexibly Ca\(^{2+}\) sensor variants with improved sensitivity (Dikici et al. 2009), defined subcellular localization (Fig. 4), or other specific features. The usage of reporter variants with particular subcellular localization might be useful to resolve the spatial distribution of Ca\(^{2+}\) patterns. Mehler and co-workers...
recently described a modular set of apoaequorin plant expression vectors that can be used to target the Ca\(^{2+}\) sensor to different subcellular compartments such as the nucleus, nucleolus, mitochondria, chloroplasts and the plasma membrane (Mehlmer et al. 2012). With such a set of expression vectors at hand, protoplast-based experiments can rapidly unravel the origin and subcellular spreading of Ca\(^{2+}\) signatures in any genetic background.

**Materials and methods**

**Plant material**

In this study we used Arabidopsis thaliana accession Col-0, Col-0 expressing cytosolic apoaequorin (line Col-0 [pMAQ2]; Knight et al. 1991), the mutants bak1-4 (At4g33430, line SALK_116202; Chinchilla et al. 2007), and bak1-4 expressing cytosolic apoaequorin (line bak1-4 [pMAQ2]; Ranf et al. 2011), the mutant fls2 (At5g66330; line SAIL_691C4; Zipfel et al. 2004) and fls2 expressing cytosolic apoaequorin (line fls2 [pMAQ2]; Frei dit Frey et al. 2012). For protoplast isolation plants were grown in soil substrate for 4 to 6 weeks at a temperature and relative humidity of 60%. For whole seedling assays, Arabidopsis Col-0 [pMAQ2] seedlings were surface-sterilized and transferred (one seed per well) to white 96-well microplates (PerlkinElmer, Waltham, MA, USA) containing 160 μl of MS basal salt medium (Murashige and Skoog 1962) supplemented with 0.25% sucrose and vitamins (Duchefa Biochemie, Haarlem, The Netherlands). The seedlings were grown for 14 days in a growth chamber with 16 h light (at 21 °C), 8 h dark (at 19 °C) before further analysis.

**Cloning of mCherry-apoaequorin and mCherry-apoaequorin-NLS fusion constructs**

The apoaequorin (AEQ) coding sequence was PCR-amplified from genomic DNA isolated from seedlings of the Col-0 [pMAQ2] line using primers AEQ1_F (5′-ATGACAGCCGACAACTACTAGT-3′) and AEQ1_R (5′-TTAGGGAGACAGCTCACCAGTA-3′). To generate the corresponding Gateway™ (www.lifetechnologies.com) entry clone, the PCR product was used as a template in a second PCR using primers AEQ_GW_F (5′-GGGCGCAACTTGTACAAAGAAAG; CAGCGTCTCATGACAGGAAACTAC-3′) and AEQ_GW_R (5′-GGGACACCTTTGACAAAGGCTGTTGAGGGACAGCTCCACC-3′). The product was inserted into the pDONR201 entry vector using the Gateway BP reaction. To generate the AEQ-NLS entry clone, the SV40 nuclear localization signal was added to the AEQ coding sequence using the primers AEQ-NLS_5′-CTATCCCTTACACCTTCTTCTTTAGGCGGAGACAGCTCCACCAGTA-3′ and AEQ1_F (above). In a subsequent PCR the Gateway recombination sequences were added to the AEQ-NLS sequence using primers: AEQ_GW_F and AEQNL_5′-GGGCGCAACTTGTACAAAGAAAG; CAGCGTCTCATGACAGGAAACTAC-3′ and AEQ1_F (above). The remaining 3′ AEQ coding sequence was subsequently inserted into the destination vector p35S-mCherry-Gateway.

**Protoplast isolation and transfection**

Protoplasts were isolated and transfected as previously described (Yoo et al. 2007). All plasmids used for transformation (p35S:mCherry–AEQ, p35S:mCherry–AEQ-NLS or FLS2p:FLS2-3xmyc–GFP) were propagated in Escherichia coli DH5α strains and purified with the NucleoBond® Xtra Midi kit (Macherey-Nagel, Düren, Germany). Transformation efficiency was calculated by counting several times the total number of protoplasts in the field of view of a microscope, and then counting the number of protoplasts showing red fluorescence due to successful transfection and resulting expression of mCherry-apoaequorin. The protoplast transfection efficiency was on average 65.8% ± 10.8% (mean ± SD).

**Quantitative Ca\(^{2+}\) measurements**

After transfection, protoplasts were stored in 2 ml batches containing approximately 4 × 10^5 protoplasts overnight at 4°C in washing and incubation solution (WI solution; 4 mM MES pH 5.7, 0.5 M mannitol, 20 mM KCl; Yoo et al. 2007) supplemented with 2 mM CaCl\(_2\) (WIc). On the following day, coelenterazine (Biosynth, Staad, Switzerland) was added to 10 μM final concentration (from a 5 mM stock solution in methanol) and protoplasts were incubated for one to four hours. Then, 100 μl of protoplast solution in WIc buffer containing approximately 2 × 10^6 protoplasts were loaded into single wells of black 96-well microplates (Perlkin Elmer, Waltham, MA, USA) and incubated 30 min in the dark before starting the Ca\(^{2+}\) measurements.

For seedlings, after 14 days the growth medium was removed and replaced by 100 μl ddH\(_2\)O containing 10 μM coelenterazine and incubated overnight in the dark. The next day, the liquid was replaced by 100 μl of ddH\(_2\)O and the seedlings were incubated 30 min in the dark before starting the Ca\(^{2+}\) measurements.

The Ca\(^{2+}\)–dependent bioluminescence was quantified in a Centro X1 LB960 or a Triton® LB 942 microplate reader (Berthold Technologies, Bad Wildbad, Germany). To determine the high resolution kinetics of the Ca\(^{2+}\) influx in protoplast suspensions and individual seedlings in single wells, the response was initiated by automatic injection of 5-fold concentrated MAMP solution (in WIc for protoplasts or ddH\(_2\)O for seedlings) to give a final concentration of 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 mM Pep1, 100 μg/ml chitin or 10 μg/ml LPS. These concentrations correspond to those used in previous studies (Zedler et al. 2004, Kwaataal et al. 2011, Ranf et al. 2011). Luminescence was continuously recorded in single wells and integrated over 1 s intervals for a total time period of at least 360 s. For IC\(_50\) determination in seedlings, instead of monitoring individual seedlings, half a microplate (48 samples) was monitored in parallel. Luminescence from each single well was integrated for 0.25 s and the cycle over all wells was repeated every 30 s. After 30 min of pre-incubation (e.g. treatment with inhibitors as described below), the response was initiated by automatic injection of 5-fold concentrated MAMP to provide a final concentration of 1 mM MgCl\(_2\) as described above, and the luminescence was recorded for additional 30 min. The second half of a microplate was used in the same manner subsequently. For each treatment, at least three technical replicates were determined (recorded subsequently) and the resulting three [Ca\(^{2+}\)] traces averaged to provide the result of one experiment. Each experiment was repeated at least 2 or 3 times yielding similar results. Given the fact that the absolute value (i.e. peak height) of the Ca\(^{2+}\) response (not so much the peak position along the time axis) was dependent on seedling size and its physiological (growth) conditions and because individual [Ca\(^{2+}\)] traces relied on recordings of single seedlings, all comparative experiments were carried out with plant material from the same growth batch.

To calculate absolute cytoplasmic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{cyt}\); the remaining aequorin in each sample (microplate well) was discharged by adding 100 μl of 2 M CaCl\(_2\) in 20% ethanol using an injector of the microplate reader). The luminescence was continuously monitored for at least 30 s in single well kinetics or repeatedly every 30 s for a period of 30 min in multiple parallel assays using half a microplate. Final [Ca\(^{2+}\)]\(_{cyt}\) were calculated as previously described (Rentel and Knight 2004).

Protoplast samples typically showed very strong luminescent signals. To avoid signal saturation, especially during normalization, protoplasts were measured in plates with black well walls, which reduces overall signal strength and improves the signal-to-noise ratio. In general, similar results were achieved when using plates with white well walls.

The elicitors used in these assays, flg22, elf18 and Pep1, were prepared as 10 mM or 1 mM stock solutions in water and stored at ~80°C. Working solutions were freshly diluted before use. The chitin solution was prepared and stored as described above (Kwaataal et al. 2011). A stock solution of 1 mg/ml of lipopoly saccharides (LPS) from Escherichia coli (L3012, Sigma, Munich, Germany) was stored at 4°C.

**Pharmacological tests and IC\(_{50}\) determination**

For IC\(_{50}\) determination, aliquots of coelenterazine-loaded protoplasts isolated from the Col-0 [pMAQ2] line or individual coelenterazine-loaded Col-0 [pMAQ2] seedlings in single wells were pre-incubated for 10 min with either staurosporine or LaCl\(_3\) (Sigma, Munich, Germany) at different final concentrations, limiting the added DMSO (as solvent for staurosporine) to <0.4% in protoplasts and <1% in seedlings. Protoplasts were pretreated with inhibitors for 10 min and seedlings for 30 min prior MAMP application. The maximum of the MAMP-triggered [Ca\(^{2+}\)]\(_{cyt}\) response of each treatment was calculated as the
percentage of corresponding control values without inhibitor. Ca^{2+} transients were compared between treatments within one experiment. The IC_{50} values were calculated following the guidelines for accurate EC_{50}/IC_{50} estimation (Sebaga 2011) using nonlinear regression to fit a 4-parameter logistic model (1) to the data, which describes a sigmoid response pattern.

\[ Y = d + \frac{a - d}{1 + (\frac{C}{c})^b} \]  

(1)

\( Y \) describes the activity as percentage of the control and \( X \) the concentration of the inhibitor. The lower and upper plateau of the curve are defined by \( a \) and \( d \), respectively, whereas parameter \( c \) is the concentration at which \( Y \) is halfway between \( a \) and \( d \). The slope factor \( b \) describes the steepness of the linear portion of the curve. For solving the equation we defined the ‘MAMP-’triggered control (+/- SD) as \( d \) and the non-induced control (+/- SD) as \( a \). The equation was solved by applying Solver (MS Excel) to the formula (1) obtaining estimates of \( a \), \( b \), \( c \) and \( d \) with the least root mean square error for the measured and corresponding calculated activities.

**Supplementary data**

**Supplementary data** are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

**References**


