Flg22-induced Ca^{2+} increases undergo desensitization and resensitization

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Abstract

The flagellin epitope flg22, a pathogen-associated molecular pattern (PAMP), binds to the receptor-like kinase FLAGELLIN SENSING2 (FLS2), and triggers Ca^{2+} influx across the plasma membrane (PM). The flg22-induced increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (FICA) play a crucial role in plant innate immunity. It's well established that the receptor FLS2 and the key downstream component, reactive oxygen species (ROS) burst, undergoes sensitivity adaptation after flg22 stimulation, referred to as desensitization and resensitization, to prevent over responses to pathogens. However, whether FICA also mount adaptation mechanisms to ensure appropriate and efficient responses against pathogens remains poorly understood. Here, we carried out detailed analyses of $[Ca^{2+}]_i$ increases upon two successive flg22 treatments, recorded and characterized, for the first time, rapid desensitization but slow resensitization of FICA in *Arabidopsis thaliana*. Pharmacological analyses showed that the rapid desensitization might be synergistically regulated by ligand-induced FLS2 endocytosis as well as the PM depolarization. The recovery of desensitized FICA might require to *de novo* FLS2 protein synthesis. FICA resensitization appeared significantly slower than FLS2 protein recovery, suggesting additional regulatory mechanisms of other components, such as flg22-related Ca^{2+} permeable channels. Taken together, we have carefully defined the FICA sensitivity adaptation, which will facilitate further molecular and genetic dissection of the Ca^{2+}-mediated adaptive mechanisms in PAMP-triggered immunity.

KEYWORDS

PAMP, flg22, FLS2, Ca^{2+} signaling, sensitivity adaptation, receptors

1. INTRODUCTION

As sessile organisms, terrestrial plants cannot escape from the everlasting intimidation of numerous microbial pathogens throughout their life cycle. To survive, plants have evolved two series of surveillance systems, the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl, 2006; Ngou et al., 2021a; Yuan et al., 2021), which are both mediated by Ca^{2+} signaling (Ngou et al., 2021b; Yu et al., 2017). PTI is initiated with the perception of conserved microbial PAMPs by their congenetic cell-surface receptors, referred to as pattern recognition receptors (PRRs). PRRs are often receptor-like kinases (RLKs), and thereby PAMP-bound RLKs activate the phosphorylational cascades, activating downstream events and ultimately leading to defense responses (Boller & Felix, 2009; Schwessinger & Ronald, 2012; Yu et al., 2017). In addition, these defense responses are well regulated/turned to allow plants to not only defend various pathogens but also match their sophisticated attack strategies that feature variable abundance and interval (Velasquez et al., 2018). Evidently, the molecular mechanisms underlying how the sensitivity of RLK-mediated processes are tightly regulated and yet deliberately turned are central to the adaptive regulation of surveillance systems.

One of the well-studied PTI surveillance systems is the bacterial flagellin-triggered immunity in plants (Liang & Zhou, 2018; Ngou et al., 2021a; Yuan et al., 2021; Zipfel, 2008). Fragment of flagellin, which contains the 22-amino acid minimal epitope flg22, binds to its receptor FLS2 and coreceptor BAK1/SERK3, and facilitates the complex formation and induction of downstream phosphorylational signaling pathway (Spoel & Dong, 2012). One of the earliest signaling events upon flg22 perception is the rapid and drastic increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Ranf et al., 2011; Yu et al., 2017). In addition to these receptor and coreceptors, several Ca^{2+}-permeable channels in the plasma membrane (PM) have been shown/suggested to be responsible for flg22-induced $[Ca^{2+}]_i$ increases (FICA), such as cyclic

nucleotide-gated channels (CNGCs) and reduced hyperosmolality-induced Ca^{2+} increase channels (OSCAs), among others (Ma & Berkowitz, 2011; Thor et al., 2020). Respectively, aequorin Ca^{2+} assay-based forward genetic screens for *changed calcium elevation* (*cce*) mutants with the phenotype of reduced FICA have also identified RLKs, such as FLS2 and botrytis-induced kinase1 (BIK1) (Ranf et al., 2014; Ranf et al., 2012). In our previous study, we observed a phenomenon of altered FICA sensitivity (Cao et al., 2017), which could point to the direction of FICA desensitization. In addition, Lammertz et al. reported that FLS2 desensitization may contribute to the *Pseudomonas syringae*-mediated attenuation of FICA (Lammertz et al., 2019), also implying the desensitization of FICA. Note that, in animals it is well known that many synchronized and measured cellular responses to extracellular stimuli/stresses, which are driven by cell-surface receptors, are dependent on the sensitivity regulation/adaption of the receptor-mediated Ca^{2+} fluxes across the PM (Hazelbauer, 2012). Therefore, we highly speculated that FICA undergo the similar sensitivity adaption, although the desensitization and resensitization of FICA have not yet been carefully and systemically studied.

The perception of flg22 by the FLS2-BAK1 receptor complex activates and releases the cytoplasmic kinase BIK1, which prompts the phosphorylation relay to active Ca^{2+} channels, leading to $[Ca^{2+}]_i$ increases (Li et al., 2014). In addition, this phosphorylation relay also triggers the influx of ions other than Ca^{2+} across the PM after a lag phase, causing extracellular alkalization and membrane depolarization (Jeworutzki et al., 2010). This depolarization may be also dependent on FICA-Ca^{2+} signaling, as it is sensitive to the inhibition of Ca^{2+} influx. FICA relay the signal directly to Ca^{2+} sensitive respiratory burst oxidase homologs (RBOHs), especially RbohD and F, the major reactive oxygen species (ROS)-generating enzymes during plant-pathogen interactions, resulting ROS burst (Waszczak et al., 2018). It is likely that ROS burst is perceived by the cell-surface H_2O_2 sensor, hydrogen-peroxide-induced Ca^{2+} increases 1 (HPCA1), which we identified recently (Wu et al., 2020). In addition, FICA activate Ca^{2+}-dependent protein kinases (CPKs), which then phosphorylate and activate RBOHs (Zhang et al.,

2018). Apart from the Ca^{2+}-dependent pathway, meanwhile, the flg22-FLS2 complex transiently activates mitogen-activated protein kinase (MAPK) cascades with a time frame similar to the ROS burst (Asai et al., 2002; Meng & Zhang, 2013; Robatzek et al., 2006; Rodriguez et al., 2010). Both of these early signaling pathways, Ca^{2+} dependent and independent, may integrate to orchestrate a complex array of transcriptional reprogramming and downstream physiological and cellular responses, and ultimately induce pathogen resistance (Yu et al., 2017). Notably, the sensitivity adaption of FLS2 protein turnover in the PM (Beck et al., 2012; Lammertz et al., 2019), membrane depolarization (Jeworutzki et al., 2010), ROS burst, and MAPK activation (Smith et al., 2014) has been studied carefully. Therefore, for better understanding of these adaption processes, it would be important to study whether and how the early Ca^{2+} signaling event, FICA, is desensitized and resensitized.

In this study, to determine the potential role of FICA-Ca^{2+} signaling adaptation in the flg22/FLS2 system in *Arabidopsis*, we adopted and established the successive flg22-elicitation procedure to detect and characterize the sensitive competency of FICA. We recorded a rapid desensitization and a slow resensitization of FICA. This adaptation of FICA was flg22-dose dependent, as expected. Interestingly, we found that the time course of FICA adaptation was related to but not well synchronized with FLS2 protein turnover in the PM. We further demonstrated that the rapid desensitization of FICA was mainly mediated by the receptor FLS2 turnover and the PM depolarization, while the slow resensitization was involved in not only the FLS2 receptor but also components other than the receptor largely, most likely Ca^{2+}-permeable channels.

2. MATERIALS AND METHODS

2.1 Plant materials and growth conditions

Arabidopsis seeds constitutively expressing aequorin in the background of Col-0 (wild type, WT) was provided by M. Knight, and these in the *bak1-4* Col-0 background was provided by G. Berkowitz (Knight et al., 1991; Ma et al., 2013). Seeds were sterilized with 2.5% plant

preservative mixture (Caisson Laboratories) and stratified at 4 ℃ for 3 days in dark and then were sown on 150 mm round Petri dishes in half-strength Murashige and Skoog (½ MS) medium containing 1.5% (w/v) sucrose, 0.8% (w/v) agar (Becton Dickinson), and 0.5% (w/v) MES, adjusted to pH 5.65 with KOH, then placed vertically in a growth chamber (16 h light/8 h dark cycle, 22 ±2 ℃, 110 μmol m^{-2} s^{-1} light intensity).

2.2. Chemicals and treatments

Unless otherwise emphasized, all chemicals were purchased from Sigma Aldrich in this study. Reconstitution of aequorin was performed *in vivo* by 10 μM coelenterazine in darkness for an indicated time (8-12 h) (Wu et al., 2020). The flg22 and elf26 peptides were synthesized by China Peptide. Elf26 was used at a concentration of 1 μM for ligand specificity analysis. Flg22 was used at a concentration of 500 nM or as indicated. Vesicular trafficking inhibitor wortmannin (Wm) was pretreated at a concentration of 30 μM in DMSO for 1 h. KCl was pretreated at indicated concentrations for 0.5 h in depolarization analysis (Allen et al., 2000; Mithofer et al., 2005). Protein synthesis inhibitor cycloheximide (CHX) was used at a concentration of 50 μM in DMSO as described (Guillaume-Schöpfer et al., 2020; Zhou et al., 2018) to analyze the effect of *de novo* protein synthesis on FICA.

2.3. Aequorin bioluminescence-based $[Ca^{2+}]_i$ analysis

Aequorin luminometry was carried out as previously described (Knight et al., 1996; Ranf et al., 2012; Wu et al., 2020; Yuan et al., 2014). For all $[Ca^{2+}]_i$ analyses, 5-day-old seedlings expressing the aequorin reporter were floated on distilled water overnight at 22 ℃ in successive light to reduce wounding response prior transferred individually to each well in 96-well plate containing 100 μL of 10 μM coelenterazine, ½ MS medium, 1% (w/v) sucrose and 0.5% (w/v) MES. Kinetic luminescence measurements were performed with an automated multimode microplate luminescence reader (Spark 10M, Tecan), and the remaining aequorin was measured by adding equal volume of discharge solution containing 3M $CaCl_2$ and 30% ethanol (Knight et al., 1996; Wu et al., 2020). Aequorin bioluminescence values were calibrated and analyzed as described previously (Knight et al., 1996; Wu et al., 2020).

2.4. Two successive rounds of flg22 elicitation

The experimental procedure of two successive rounds of flg22 elicitation was adopted from previous studies with modifications (Smith et al., 2014). To allow direct comparisons, the aequorin-reconstituted seedlings were performed in the same 96-well plate and elicited with the 1st round of flg22 elicitation and detected $[Ca^{2+}]_i$ increases. These same seedlings were washed with water, then re-elicited with the 2nd round of flg22 at a indicated concentration and at an indicated interval for desensitization and resensitization analyses, and recorded $[Ca^{2+}]_i$ increases. The time course of sensitization was analyzed by the difference of flg22-induced $[Ca^{2+}]_i$ increases (Δ $[Ca^{2+}]_i$) with variable intervals between these two treatments. The competency of sensitization of Ca^{2+} was calculated by the relative $[Ca^{2+}]_i$, which is the ratio between the 2nd round of $[Ca^{2+}]_i$ peak changes to the 1st round of $[Ca^{2+}]_i$ peak changes.

2.5. Statistical analysis

Each independent experiment was performed at least three times with similar results. The statistical analysis was performed using Office 365 Excel software (Microsoft). Data were presented as mean ± s.d./s.e.m. For all bar graphs, *P* values were calculated by two-sided Student's *t*-test (*t*-test). To analyze the difference between genotypes in curve/line graphs two-way analysis of variance (ANOVA) was carried out. Values of $P < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Desensitization and resensitization of flg22-induced $[Ca^{2+}]_i$ increases (FICA)

It is well established that the receptor-mediated desensitization is an important regulatory feature that prevents overstimulation and allows for the relative linear response range to the ambient stimulation level in animals (Gainetdinov et al., 2004). Similar to animals, after the receptor-mediated perception of PAMPs during plant PTI defense, the sensitization and desensitization of this signaling pathway occurs, allowing plants to respond accordingly to persistent pathogen invasion (Velasquez et al., 2018). Flg22 is known to activate Ca^{2+} influx currents and trigger

$[Ca^{2+}]_i$ increases in a receptor-dependent manner in planta (Jeworutzki et al., 2010; Ranf et al., 2011). Based on these regulatory mechanisms underlying the adaptation of several components in the flg22 signaling pathway (Vetter et al., 2012), we speculated that FICA should also undergo the desensitization and resensitization.

First, we carefully analyzed the dose-dependent response of FICA using aequorin luminometry in aequorin-expressing *Arabidopsis* seedlings as we carried out previously (Wu et al., 2020). The increases in peak $[Ca^{2+}]_i$ (Δ $[Ca^{2+}]_i$) were dependent on the concentration of flg22 that was applied (Figure 1a). Significant $[Ca^{2+}]_i$ increases were detected at the concentration above 0.05 nM, and the saturating $[Ca^{2+}]_i$ response was showed above 1,000 nM. From the fitting curve, an apparent effective concentration (EC_{50}) of 98.82 ± 28.68 nM was calculated, which is within the range observed for FICA in previous studies (Jeworutzki et al., 2010). Therefore, we decided to use 500 nM flg22 as the reference concentration in subsequent analyses. Note that, under our analysis conditions, the $[Ca^{2+}]_i$ of aequorin-expressing *Arabidopsis* seedlings (WT) in the resting state was 88 ± 3 nM, which was consistent to these reported previously (Stael et al., 2012).

We established the assay of two successive rounds of flg22 elicitation to examine desensitization and resensitization of FICA as illustrated in Figure 1b. The seedlings were first treated with 500 nM flg22, then washed using water, and treated with the 2nd round of 500 nM flg22 at a 0.5 h interval time (green). In comparison to the control with water as the 1st treatment (Figure 1c), FICA showed a dramatic reduction during the repeated application of flg22 (Figure 1d), showing clearly that FICA were desensitized (red). The Δ $[Ca^{2+}]_i$ peak was only 0.03 ± 0.002 µM in the repeatedly flg22 treatment compare with 0.19 ± 0.028 µM in the first treatment or 0.17 ± 0.014 µM in the control, having an about 6-fold reduction. The Δ $[Ca^{2+}]_i$ peak occurred approximately 3 min in the 2nd flg22 treatment, while this peaking time was around 1 min in the control or the 1st round of flg22 treatment. However, when the interval time was extended to 24 h between the 1st and 2nd flg22 treatment, the Δ $[Ca^{2+}]_i$ showed typical resensitization (Figure 1e, blue). FICA were significantly elevated within 1 min for the 2nd flg22 application although the Δ $[Ca^{2+}]_i$ showed slightly lower amplitudes and shorter duration compared with these in the 1st round of flg22 stimulation. Taken together, as expected these data showed the phenomena of desensitization and resensitization of FICA.

Next, to better understand the desensitization and resensitization of FICA, we should determine their kinetics. Then, we tested a series of interval time between the two flg22 treatments as illustrated in the scheme in Figure 2a. The desensitization of FICA occurred very fast, within about half an hour, after the 1st flg22 elicitation; however, the resensitization of FICA required a much longer time, over 10 hours, to develop after the 1st elicitation (Figure 2a). Specifically, after the initial elicitation of flg22, FICA desensitized within 30 min, having an ~80% reduction (Figure 2b), maintained at the low plateau level for 1-2 h (desensitization phase, red), and recovered ~60% from the lowest point at the interval of 24 h (resensitization phase, blue; Figure 2a). It is worth noting that at the 16 h interval time, the relative $\Delta [Ca^{2+}]_i$ was 0.35 r.u., beginning to show a significant recovery capacity. It appeared the time constant for resensitization $\tau_R \approx 20$ h. In the control with water as the 1st round of treatment (0 nM flg22), the relative $\Delta [Ca^{2+}]_i$ seemed to have no drastic change with the increase of interval time, and maintain above 0.9 r.u., near the level of the initial flg22 elicitation (Figure 2a). Note that, the solution that was injected into the wells of 96-well-plate during Ca^{2+} measurement induced $[Ca^{2+}]_i$ increases, likely caused by the mechanical stimulation and hypoosmotic stress, among others. Nevertheless, the minimal effect of 0 nM flg22 on FICA (Figure 2a, gray line) on $\Delta [Ca^{2+}]_i$ showed that the desensitization and resensitization of FICA are independent of these mechanical and hypoosmotic stresses under the imposed conditions. In other words, the desensitization and resensitization of FICA was specific to flg22.

To increase time resolution for the rapid desensitization of FICA, we added a few time points between the two rounds of elicitation (Figure 2b). Compared with the initial flg22 treatment, the $\Delta [Ca^{2+}]_i$ evoked by the 2nd round of flg22 elicitation was approximately 0.054 μM within 20 min intervals, about one-third of the initial flg22 elicitation (0.180 μM). It is clear the time constant for desensitization $\tau_D < 15$ min. The $\Delta [Ca^{2+}]_i$ decreased to the plateau at the interval of 0.5 h and maintained around 0.030 μM for 1 h (Figure 2b). To further determine the competency of FICA, we assessed the $\Delta [Ca^{2+}]_i$ of two rounds of flg22 elicitation at intervals of 0.5 h and 48 h, which represent the greatest degree of desensitization and resensitization, respectively (Figure 2c). The $\Delta [Ca^{2+}]_i$ of desensitization and resensitization were 0.14 r.u. and 0.90 r.u., about a 6-fold difference, in respected to the control value of 1, respectively (Figure 2c). Taken together, our results showed that FICA underwent rapid desensitization (to ~14%)

and slow resensitization (to ~90%). Thus, we demonstrated, for the first time to our knowledge, the desensitization and resensitization of FICA.

3.2. FICA desensitization and resensitization are flg22-dose dependent

It is well known that desensitization and resensitization of PM receptors and ion channels are dependent on the concentration of ligands (Sanz-Salvador et al., 2012; Shankaran et al., 2007). To determine whether the FICA sensitivity adaptation is also in a dose-dependency manner, we treated seedlings with flg22 at the variable concentrations ranging from 0.05 to 500 nM in the 1st round, while used constantly 500 nM flg22 in the 2nd round at the same interval of 0.5 h for desensitization analysis or at 16 h for resensitization analysis as illustrated in Figure 3a. With the increase in the concentration of flg22 applied in the 1st round, FICA gradually increased from 0.04 μM to 0.19 μM (Figure 3a, black traces). The notable FICA peaks began to appear at 0.5 nM flg22, the time to the peak gradually became shorter, and the duration lasted longer (Figure 3a). The $\Delta [Ca^{2+}]_i$ evoked by 2nd flg22 elicitation at 0.5 h gradually decreased with the increase in concentration of flg22 applied in the 1st round (Figure 3a, red traces), from 0.17 μM to 0.03 μM. With the interval time extended to 16 h, FICA similarly decreased from 0.17 μM to 0.05 μM (Figure 3a, blue trances). In addition, the timing to peak appeared also dependent on the concentration of flg22 applied in the 1st round, i.e. the peaks occurred later with the increase in flg22 concentrations.

Quantitative analyses showed the dose-dependent desensitization of FICA with an EC_{50} of 1.49 ± 0.18 nM (Figure 3b). The resensitization competency showed an inverse correlation with flg22 concentrations with an EC_{50} of 3.08 ± 0.36 nM (Figure 3c). The EC_{50} values for desensitization and resensitization are in the similar range, suggesting these two processes are highly coordinated. Our results showed that the desensitization and resensitization of FICA are dose dependent. Interestingly, the minimum flg22 concentration, which is required to trigger the FICA sensitivity adaptation, is probably at the nanomolar range, which is much lower than that reported previously for triggering the internalization of FLS2 (Beck et al., 2012; Lammertz et al., 2019), suggesting the underlying mechanisms other than FLS2 might also be profoundly involved in the FICA sensitivity adaptation.

3.3. Depolarization may be involved in FICA desensitization

Upon flg22 perception by receptors, the influx of Ca^{2+} and H^+ ions is often accompanied by the efflux of Cl^-, NO_3^-, and K^+ ions, and these ion fluxes collectively depolarize the plasma membrane potential (Jeworutzki et al., 2010; Yu et al., 2017). Previous studies have shown that the treatment of plants with about 20 mM to 100 mM KCl cause the membrane depolarization (Allen et al., 2000; Mithofer et al., 2005). To determine the possible effect of depolarization on the FICA desensitization, we pre-treated seedlings with KCl. First, we optimized the incubation time and concentration of KCl, and found that preincubation of seedlings with KCl at concentration ≤ 50 mM for 0.5 h did not affect the kinetics of FICA, as there was not large alteration in the time, amplitude, and duration of $\Delta [Ca^{2+}]_i$ in the 1st round of flg22 elicitation (Figure 4a, b). Note that, for pharmacology treatments, we obtained the ranges of drug concentration and treatment time from the literature, optimized these two parameters in our system via small scale trails, and then carried out experiments that are shown in the figures using these two optimized parameters under well controlled conditions. The $\Delta [Ca^{2+}]_i$ was 0.188 μM in seedlings pre-treated with KCl, which was similar to the control of 0.184 μM in seedlings preincubated with water (Figure 4b). With the 2nd round of elicitation, the $\Delta [Ca^{2+}]_i$ was higher in KCl-pretreated seedlings than in the control seedlings (Figure 4a). Note that, the FICA decay was also inhibited clearly by KCl treatment, however, the 2nd FICA were still much large under KCl treatment compared to the control.

After preincubation with KCl, with the increase of the interval times between the two rounds of elicitation, FICA in the 2nd round were decreased slowly from 0.122 μM to 0.095 μM within 1 h, compared with the control elicited with water in the 1st round (Figure 4c), but still had ~60% remained at an interval of 1 h. The near linear trend of FICA with KCl preincubation did not correlate with that of water preincubation, and the higher FICA value of 0.56 r.u. showed a dramatic decrease in the desensitization of FICA (Figure 4d). Furthermore, to verify this effect of KCl, we used a lower concentration of 30 mM KCl, and observed similar but smaller inhibitor effect on the desensitization of FICA (Figure 4b, d). Based on these results, we conclude that the PM depolarization might be associated with the FICA sensitivity adaptation.

3.4. FICA desensitization is flg22-ligand specific dependent and may require flg22-receptor association

It is also well known that desensitization and resensitization of receptors and ion channels in the PM are ligand-specific dependent (Hedrich, 2012; Robatzek et al., 2006). To probe the ligand-specificity of FICA adaptation, we used another PAMP, elongation factor Tu (EF-Tu, elf) from bacterial pathogens, which elicits the first line of defense of PTI, including Ca^{2+} signaling similar to flg22 (Zipfel et al., 2006). Previous study shows that in contrast to flg22, the conserved 26-amino acid peptide from EF-Tu, elf26, could not remove the receptor FLS2 from the PM after perception by the EF-Tu RECEPTOR (EFR) (Smith et al., 2014). To determine the ligand specificity, we determined the optimum concentration of elf26 as 1 μM, at which elf26 could elicit increases in $[Ca^{2+}]_i$ similar to these of FICA induced by 500 nM flg22 (Figure 5a, b). Specifically, with the 1st round of 1 μM elf26 elicitation, the time to Δ $[Ca^{2+}]_i$ peak was about 4 min, and the amplitude reached 0.18 μM, which were similar to FICA with 500 nM flg22 elicitation (Figure 5a, b). After the first elicitation with 1 μM elf26, the amplitude of Δ $[Ca^{2+}]_i$ reduced slightly to 0.16 μM with the 2nd round of 500 nM flg22 elicitation at the 0.5 h interval, although the time to peak was still ~ 1 min (Figure 5a). We then analyzed carefully the effect of non-specific ligands on FICA desensitization. The elf26 treatment in the 1st round indeed reduced slightly FICA, while the mild desensitization was recovered largely with increases in intervals within 1 h (Figure 5c), in contrast to 20 h for flg22. Quantitative analysis showed that compared to these with 500 nM flg22 application in the 1st round as shown in Figure 2b, the elf26-caused desensitization was much smaller and recovered much faster (Figure 5d).

To examine if flg22-receptor association is likely involved, we tested the effect of BAK1, the coreceptor of FLS2, which does not undergo endocytosis after the recognition of flg22, in contrast to FLS2 (Sun et al., 2013). In the *bak1* mutant seedlings, although FICA were substantially smaller, there was no significant difference in flg22 reelicitation within the 20 min interval, however, FICA were significantly higher than these of WT with a 30 min interval (Figure 5e). Quantitative analysis showed that *bak1* had much larger relative FICA compared to WT (Figure 5f), suggesting that the mutation in BAK1 might affect somehow FICA desensitization. These results showed that the desensitizing adaptation of FICA might be ligand-specific and seemed to be involved in the activation of FLS2, consistent with the notion that the

ligand removes FLS2 from the cell surface during the ligand perception and down-regulates FLS2 sensitivity (Beck et al., 2012; Lammertz et al., 2019).

3.5. FICA desensitization and resensitization may require largely FLS2 protein trafficking and *de novo* synthesis, respectively

Increasing evidence indicates that the subcellular localization and vesicle trafficking routes of cell-surface PRRs lead to the internalization of receptor complexes, which are either recycled back to the cell surface or targeted to the vacuole for degradation, fine tuning the signal initiation, attenuation, and adaption (Claus et al., 2018; Robatzek et al., 2006). The cooperative relationship between flg22-induced endocytosis/degradation of FLS2 and these PTI-triggered early signaling events has been investigated using genetic and pharmacological approaches. For instance, the clathrin-dependent endocytosis of activated FLS2 from the FLS2-BAK1 receptor complex after flg22 elicitation is followed by its internalization into late endosomal compartments destined for vacuolar degradation at 30-60 min (Beck et al., 2012; Lammertz et al., 2019; Mbengue et al., 2016). It has been reported that the flg22-induced endocytotic transport of FLS2 in the PM is bidirectional, undergoes constitutive recycling and internalization, and is inhibited by wortmannin (Wm), a vesicular trafficking inhibitor of phosphoinositide 3-kinases (PI3Ks) and PI4Ks (Geldner & Robatzek, 2008).

Next, we used Wm to determine the effect of endocytosis of FLS2 on the adaption of FICA. Our results showed that 30 μM Wm preincubation for 1 h did not alter the time and duration of the $\Delta [Ca^{2+}]_i$ of FICA although reduced the amplitude slightly (Figure 6a, b). After washout of flg22 applied in the 1st round, remarkably we observed only a slightly reduced $\Delta [Ca^{2+}]_i$ in the 2nd round of flg22 elicitation with Wm treatment (Figure 6a, + Wm, light red circle), in contrast to the drastic FICA reduction without Wm treatment (Figure 6a, – Wm, black circle), showing that Wm inhibited FICA desensitization. Detailed analyses of varied interval times confirmed that with Wm preincubation, the $\Delta [Ca^{2+}]_i$ evoked by the flg22 re-elicitation was slightly but consistently lower than that of the control (Figure 6c), but significantly higher than those without Wm preincubation (Figure 6d), showing greatly reduced desensitization of FICA. The Wm effect indicates that the endocytosis of FLS2 might play a key role in the desensitization of FICA. Note that, the prolonged decreases in $[Ca^{2+}]_i$ detected with Wm

incubation for 1 h suggest that the desensitization of FICA might also be regulated by other mechanisms.

Our data have shown that the time course of the FICA sensitivity adaptation is inconsistent with that of the constitutively recycling of FLS2 in the PM and the recovery of the resting state from flg22-triggered membrane depolarization (Jeworutzki et al., 2010; Smith et al., 2014). To reveal further insights into the mechanism along this line of FLS2, we determined if the *de novo* synthesis of FLS2 protein is involved in FICA resensitization. Cycloheximide (CHX) is a broadly used inhibitor for protein *de novo* synthesis, and has been used to study FLS2 turnover (Zhou et al., 2018) as well as the resting $[Ca^{2+}]_i$ (Guillaume-Schöpfer et al., 2020). As CHX has pleiotropic inhibitory effects on the translation of large numbers of proteins, we adjusted the CHX concentration and preincubation time to minimize the potential effect of CHX on the background abundance of FLS2 in the PM. We found that preincubation of seedlings with 50 µM CHX for 1 h before the 1st round of 500 nM flg22 elicitation did not alter the amplitude of FICA, but delayed the time to peak and extended the duration compared to the control preincubated with DMSO (Figure 7a). FICA were 0.18 µM with CHX preincubation, identical to that with DMSO preincubation (Figure 7b). After the 1st round of treatment, we washed the seedlings with water and incubated them with the same concentration of CHX for 2 h, rewashed, and conducted the 2nd round of flg22 elicitation at the interval of 24 h (see the scheme, Figure 7a). Compared with the control, the amplitude of the 2nd round FICA was greatly reduced, and the time to peak was delayed by ~2 min, almost doubled (Figure 7a). Subsequently, we only treated seedlings with 50 µM CHX for 2 h during the interval time and washed out CHX (Figure 7c). Although the $\Delta [Ca^{2+}]_i$ with the 2nd round elicitation was increased from 0.021 µM at 4 h to 0.079 µM at 24 h for CHX treatment and from 0.043 µM to 0.145 µM for DMSO control, it significantly reduced at all tested intervals for CHX-treated seedlings (Figure 7c). Quantitative analyses showed that the smaller recovery coefficient of 0.207 ± 0.095 with CHX incubation compared to 0.286 ± 0.069 with the DMSO control also indicated the decrease in the resensitization of FICA under CHX treatment (Figure 7d). These results suggest that the newly synthesized proteins, most likely FLS2, might be required for the resensitization process of FICA. Note that, it has been shown that CHX triggered an increasse in the resting $[Ca^{2+}]_i$ (from

~50 nM to ~85 nM) (Guillaume-Schöpfer et al., 2020), which might be related to the slowed FICA decay and the CHX inhibitory effect on FICA resensitization.

4. DISCUSSION

Receptor-mediated PAMP perception leads to a broad spectrum of immune responses with spatial and temporal dynamics, and the Ca^{2+} influx is one of the earliest and essential signaling events with unique spatial and temporal signatures (Yu et al., 2017). In this study, we adopted/developed experimental procedures to systematically analyze increases in $[Ca^{2+}]_i$ to successive flg22 elicitations. We recorded and characterized, for the first time to our knowledge, the sensitive adaption of FICA in detail, which features a rapid desensitization and a slow resensitization. In addition, we determined the ligand-, time-, and dose-dependence of FICA. Furthermore, as expected we found that FICA sensitive adaption required FLS2 activation and turnover in the PM. Finally, from these results we speculated that Ca^{2+} permeable channels might also play a key role in the adaption of FICA.

4.1. Desensitization and resensitization of FICA do occur

The sensitive adaption of several components in the flg22 signaling pathway have been studied as summarized in Table S1. For instance, the decreases in FLS2 protein level or the increases in FLS2 endosomal spots are observed primarily at 20-60 min after flg22 treatment, which were shown by the live-cell imaging and biochemical analyses (Beck et al., 2012; Cui et al., 2018; Lammertz et al., 2019; Smith et al., 2014). The *de novo* synthesis of FLS2 occurs after 2 hours, which may prepare for a new round of flg22 perception at least in respect to the MAPKs activation and ROS burst (Smith et al., 2014). These studies have shown FLS2 turnover in the PM, which plays an essential role in the adaption of flg22 signaling pathway in general. In this study, we recorded and characterized the desensitization and resensitization of FICA, which should also be physiological relevance. The resetting sensitivity of PAMP-triggered Ca^{2+} signaling allows plants to avoid an over-mobilization of internal and external stores of Ca^{2+}, and to prevent the plants' overreaction to the invasion of persistent pathogens (Leblanc-Fournier et

al., 2014). For example, Yoshioka et al. reported a rare gain-of-unction *constitutive expresser of PR genes22* (*cpr22*) mutant almost 20 years ago, which exhibited multiple autoimmune phenotypes and Ca^{2+}-dependent spontaneous cell death (Yoshioka et al., 2001; Yoshioka et al., 2006). These altered defense responses in *cpr22* were resulted from constitutively activated Ca^{2+} influx, which was caused by the functional chimeric CNGC (AtCNGC11/12) due to a deletion mutation (Dietrich et al., 2020). It would be interesting to examine the adaption of FICA in *cpr22*. Overall, through the sensitivity adaptation of effective elements, plants alter their competency to respond to recurrent stimuli at the molecular, cellular, or tissue levels to avoid overturning the immune output (Mauch-Mani et al., 2017), in which FICA should function in concert with these effective elements.

Ca^{2+} is a universal second messenger in animals and plants, and almost all biotic and abiotic stresses trigger Ca^{2+} increases in plants (Behera et al., 2018; Berridge et al., 2003; Hetherington & Brownlee, 2004; Kudla et al., 2018; Lamers et al., 2020; Oldroyd, 2013; Ranf, 2017; Yu et al., 2017). Interestingly, previous studies have reported the sensitivity adaptation of abiotic stress-triggered Ca^{2+} signaling (Knight et al., 1996; Knight et al., 1992). For example, after the initial Ca^{2+} transient in response to cold shock or wind treatment, the observed attenuation of Ca^{2+} signaling was seen in repetitive stimuli and the regained resilience to a new round of stimulation within a few minutes, in contrast to many hours for FICA. Although the molecular mechanisms remain to be carefully elucidated for the biotic and abiotic Ca^{2+} signaling, the adapted Ca^{2+} signaling by acclimation suggests that it plays a central role in the stress priming.

4.2. FICA feature rapid desensitization but very slow resensitization

The sensitivity adaptation is often associated with a fast desensitization response and a slower resensitization response (Piper et al., 2005). We have also observed the similar sensitivity adaption for FICA. In Table S1, for a comparison we have carefully summarized the time course of the sensitivity adaptation and coupling relationship of components in the flg22 signaling pathway. Clearly, the resensitization time for FICA is much longer (16-48 h) than these (~2 h) for the PM depolarization, ROS burst, MAPK activation, and FLS2 turnover (Table S1). It is possible that mechanisms regulating these signaling components may differ. For example, BIK1

and PBLs, belonging to the RLCK VII subfamily, play a dominant role in the flg22-triggered Ca^{2+} influx and ROS production (Tang et al., 2017). However, loss-of-function mutants of BIK1 and PBL1, and other higher-order RLCK VII subfamily members do not show significantly compromise in flg22-triggered MAPK activation (Rao et al., 2018). A recent study showed that another RLCK subfamily XII member BRASSINOSTEROID-SIGNALING KINASE1 (BSK1), which is also one of the key components in the FLS2 receptor complex, directly phosphorylates MAPKKK5 to regulate immunity (Yan et al., 2018). These studies indicate that the FLS2/flg22 system may activate different downstream signaling events by specific RLCK members. In addition to FLS2 turnover for FICA adaption, we speculate that another regulatory layer might be the transition from an inactivated to a close state of Ca^{2+} permeable channels, which might take a longer time. CNGC19 and CNGC20 have been proposed to play a role in immunity, wound signaling, and insect resistance (Dietrich et al., 2020). A recent study has revealed a novel mechanism of CNGC regulation in protein stability, in which BAK1/SERK4 phosphorylated CNGC19/20 and accelerated the channel turnover (Yu et al., 2019). Therefore, it is plausibly that as many components as well as complex mechanisms are involved in FICA sensitivity adaptation, this process may require a longer time to complete.

4.3. FICA sensitive adaption may require FLS2 activation and turnover

Similar to the sensitization of animal membrane receptors/channels, such as ligand-gated ion channels, G-protein-coupled receptors, and tyrosine kinase receptors, the endocytosis of FLS2 plays an essential role in altering sensitivity adaptation of several key components in the flg22 signaling pathway as mentioned above (Sorkin & Von Zastrow, 2009; Tang et al., 2017), as well as the FICA adaptation showed in this study. Vesicular trafficking of FLS2 negatively regulates Ca^{2+} influx, ROS burst, stomatal closure, and callose deposition, however, there is no apparent effect on the MAPK activation (Ben Khaled et al., 2015; Rodriguez et al., 2010; Yu et al., 2017). Furthermore, the decrease in the abundance of FLS2 by endocytic degradation is considered to reduce ROS production and MAPKs phosphorylation, lowing the host cell sensitivity toward encountering further flg22 stimuli, and the subsequent *de novo* synthesis of FLS2 could contribute to the resensitization of flg22 signaling (Smith et al., 2014). Interestingly, we observed that the flg22 concentration required for triggering FICA desensitization was much lower than

that for inducing FICA itself (Figure 2). The complete activation of FLS2 might not be required for the FICA desensitization, considering that the amount of flg22-bound FLS2 was limited. In contrast, flg22-induced Ca^{2+} elevation and FLS2 degradation appeared to be more dependent on the amount of flg22-bound/activated FLS2 to signal the downstream components, such as RLCKs, PBLs, RBOH, and ion channels (Boller & Felix, 2009). Alternative explanations might be related to variations in these downstream components. It is also possible that the inactivation of these components could lead to the desensitization of Ca^{2+} signaling. The critical RLCK BIK1 and its closest homolog PBLs, directly interact with FLS2 are required for activating the downstream components, such as OSCA1.3/1.7 channels (Thor et al., 2020). BIK1/PBLs are indicated as rate-limiting components in PTI signaling and dynamically fine tune the degree of immune output (Liang & Zhou, 2018). Especially for BIKI, which differs from the polyubiquitination regulated by PUB25 and PUB26, the monoubiquitination of BIKI induced by flg22 facilitates the dissociation of BIKI from the FLS2, which precedes FLS2 endocytosis (Ma et al., 2020). The phosphorylation state and/or stability of BIK1 are also negatively regulated by Ca^{2+}-dependent protein kinase CPK28, which may be further activated through the PTI-induced Ca^{2+} influx (Monaghan et al., 2014). Therefore, this negative feed-back regulation module may play an important role in the sensitive adaption of FICA.

Previous studies have shown that the *de novo* synthesis of FLS2 occur after 2 hours of flg22 treatment, which affects the MAPKs re-activation and/or PM re-depolarization in response to additional flg22 stimulation (Jeworutzki et al., 2010; Smith et al., 2014). Our results on FICA resensitization were in line with these previous reports that the resensitization process not only relies on the *de novo* synthesis of FLS2 but also is affected by other modules like BIK1. Our data showed only small FICA after 2 h (Figure 2a), and the resensitization rate was inhibited by CHX treatment (Figure 7).

4.4. Fitting FICA adaption into the pathogen Ca^{2+} signaling network

In this study, we have added a missing piece of Ca^{2+} signaling into the sensitive adaption of the flg22 signal transduction pathway. Certainly, our characterization of FICA adaption will provide a framework for dissecting the molecular mechanisms underlying not only flg22- but also other PAMP/DAMP-induced $[Ca^{2+}]_i$ increases. For instance, we could test how these known Ca^{2+}-

permeable channels (Demidchik et al., 2018), which are thought to function in the flg22 Ca^{2+} signaling, such as CNGCs, OSCAs or GLRs, contribute to the FICA sensitivity adaptation by analyzing their corresponding genetic mutants using a candidate approach (Behera et al., 2018; Jacob et al., 2021). In addition, given that the previous forward genetic screens based on FICA have only identified the receptor and its associated RLK receptors without the success for the long sought-after Ca^{2+} channels (Ranf et al., 2014), new genetic screens could be redesigned based on the FICA adaption to allow us to identify novel components, similar to these reported previously (Choi et al., 2014; Jiang et al., 2019; Wu et al., 2020; Yuan et al., 2014). Furthermore, the interaction between the two Ca^{2+} signaling networks in PTI and ETI could be examined by monitoring FICA resensitization process. In pathogen-induced ETI, the Ca^{2+} influx and ROS burst occur 4-6 h after pathogen infection, in contrast to their fast paces (in min) in PTI (Ngou et al., 2021b; Yu et al., 2017), while the ETI-mediated Ca^{2+} influx lies within the resensitization phase of FICA. Recently, we have identified that the helper nucleotide-binding-leucine-rich-repeat-receptor (NLR) proteins NRG1 and ADR1 form Ca^{2+}-permeable channels upon pathogen infection, which are responsible for the ETI Ca^{2+} influx (Jacob et al., 2021). Remarkably, similar results were observed for another NLR protein ZAR1 (Guozhi et al., 2021), implying a broad Ca^{2+}-mediated function of NLRs (Ngou et al., 2021a; Ngou et al., 2021b; Yuan et al., 2021). Certainly, the study on the molecular mechanisms of how FICA is adapted as well as integrated in concert with other sensitivity adaptation processes in PTI and ETI will further our understanding of how plants respond to diverse pathogens, which are equipped with various spatiotemporal attacking modes under natural environments.

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AUTHOR CONTRIBUTIONS

This project was conceptualized by Feihua Wu and Zhen-Ming Pei. Yuan Chi, Chao Wang, Mengyun Wang, Di Wan, and Feifei Huang carried out initial experiments for aequorin analysis and system optimization. Yuan Chi and Feihua Wu designed and conducted aequorin analysis, data processing and statistical analysis. Zhonghao Jiang, Bridget M. Crawford, Tuan Vo-Dinh, and Fang Yuan participated the development of Ca^{2+} measurements and data interpretation. All authors provided technical supports and/or contributed to the interpretation of results. The paper was written by Yuan Chi, Feihua Wu and Zhen-Ming Pei. All authors approved the final manuscript.

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