



**HAL**  
open science

# **Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana***

Mark Kwaaitaal, Rik Huisman, Jens Maintz, Anja Reinstädler, Ralph Panstruga

► **To cite this version:**

Mark Kwaaitaal, Rik Huisman, Jens Maintz, Anja Reinstädler, Ralph Panstruga. Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana*. *Biochemical Journal*, 2011, 440 (3), pp.355-365. 10.1042/BJ20111112 . hal-00658154

**HAL Id: hal-00658154**

**<https://hal.science/hal-00658154>**

Submitted on 10 Jan 2012

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**IONOTROPIC GLUTAMATE RECEPTOR (iGluR)-LIKE CHANNELS MEDIATE MAMP-INDUCED CALCIUM INFLUX IN *ARABIDOPSIS THALIANA*****Mark Kwaaitaal<sup>\*</sup>, Rik Huisman<sup>\*</sup>, Jens Maintz<sup>\*</sup>, Anja Reinstädler<sup>\*</sup> and Ralph Panstruga<sup>\*,§</sup>**<sup>\*</sup> Max-Planck Institute for Plant Breeding Research, Department of Plant-Microbe Interactions, Carl-von-Linné-Weg 10, 50829 Köln, Germany<sup>§</sup> RWTH Aachen University, Institute for Biology 1, Unit of Plant Molecular Cell Biology, 52056 Aachen, GermanyAddress correspondence to Ralph Panstruga, RWTH Aachen University, Institute for Biology 1, Unit of Plant Molecular Cell Biology, Worringer Weg 1, 52056 Aachen, Germany, Tel. +49 (0) 241 8026655, Fax. +49 (0) 241 8022637, Email: [panstruga@biol.rwth-aachen.de](mailto:panstruga@biol.rwth-aachen.de)**Running head:** iGluR-like channels mediate MAMP-triggered calcium influx in *Arabidopsis***Key words**

Calcium channel, calcium signature, elicitor, ionotropic glutamate receptor channel, microbe-associated molecular pattern, plant immunity

**Abbreviations**

CICR	calcium-induced calcium release
CNGC	cyclic nucleotide-gated channel
HR	hypersensitive response
iGluR	ionotropic glutamate receptor
MAPK	mitogen-activated protein kinase
ROS	reactive oxygen species
RR	Ruthenium Red
VGCC	voltage-gated calcium channel

## Synopsis

Binding of specific microbial epitopes (microbe-associated molecular patterns, MAMPs) to pattern recognition receptors (PRRs) and subsequent receptor kinase activation are key steps in plant innate immunity. One of the earliest detectable events after MAMP perception is a rapid and transient rise in cytosolic calcium ( $\text{Ca}^{2+}$ ) levels. In plants, knowledge about the signaling events leading to  $\text{Ca}^{2+}$  influx and on the molecular identity of the channels involved is scarce. We used a transgenic *Arabidopsis thaliana* line stably expressing the luminescent aequorin  $\text{Ca}^{2+}$  biosensor to monitor pharmacological interference with  $\text{Ca}^{2+}$  signatures following treatment with the bacterial peptide MAMPs flg22 and elf18 and the fungal carbohydrate MAMP chitin. Using a comprehensive set of compounds known to impede  $\text{Ca}^{2+}$  transport processes in plants and animals we found strong evidence for a prominent role of amino acid-controlled  $\text{Ca}^{2+}$  fluxes, probably through ionotropic glutamate receptor (iGluR)-like channels. Interference with amino acid-mediated  $\text{Ca}^{2+}$  fluxes modulates MAMP-triggered mitogen-activated protein kinase (MAPK) activity and affects MAMP-induced accumulation of defense gene transcripts. We conclude that the initiation of innate immune responses upon flg22, elf18 and chitin recognition involves apoplasmic  $\text{Ca}^{2+}$  influx via iGluR-like channels.

## Introduction

Eukaryotic organisms possess an extensive array of receptor proteins to sense their environment, including the presence of benign or hostile (micro-) organisms. Major pathways for the relay of exogenous signals to the cytoplasm/nucleus invoke pattern recognition receptors (PRRs) that sense specific microbial epitopes termed microbe-associated molecular patterns (MAMPs). PRRs play a key role in plant innate immunity by the activation of defense response pathways upon MAMP sensing (MAMP-triggered immunity) [1]. Examples of PRRs comprise FLAGELLIN SENSING2 (FLS2), ELONGATION FACTOR-Thermo unstable (EF-Tu) RECEPTOR (EFR) and CHITIN ELICITOR RECEPTOR KINASE1 (CERK1). FLS2 and EFR activate downstream signaling after detection of 22 and 18 amino acid-long epitopes of bacterial flagellin (flg22) and EF-Tu (elf18), respectively [2, 3]. CERK1 is essential for the recognition of chitin polymers, major constituents of the fungal cell wall [4]. Detection of MAMPs by PRRs typically results in a series of early cellular responses such as fluxes of calcium ( $\text{Ca}^{2+}$ ) and other ions, the generation of reactive oxygen species (ROS), the initiation of mitogen-activated protein kinase cascades (MAPK(K/KK)) and the activation of  $\text{Ca}^{2+}$ -dependent protein kinases (CDPKs) [1]. Despite their canonical occurrence, the functional role of these archetypal responses in plant innate immunity remains largely obscure.

$\text{Ca}^{2+}$  acts as a second messenger in response to many biotic and abiotic stimuli, e.g. microbial attack, symbiosis, heat shock, cold shock, osmotic stress, oxidative stress, light conditions and abscisic acid [1, 5, 6]. These different cues each induce their own specific  $\text{Ca}^{2+}$  signatures; transients that differ in timing as well as the number and magnitude of  $\text{Ca}^{2+}$  spikes. The transient increase of cytosolic  $[\text{Ca}^{2+}]$  results from passive  $\text{Ca}^{2+}$  influx from the apoplast or intracellular  $\text{Ca}^{2+}$  stores through dedicated channels [7]. Since  $\text{Ca}^{2+}$  is cytotoxic, low concentrations are maintained in the cytosol and nucleus of resting cells.  $\text{H}^+/\text{Ca}^{2+}$ -exchangers and ATP-dependent  $\text{Ca}^{2+}$  pumps ( $\text{Ca}^{2+}$ -ATPases) expel  $\text{Ca}^{2+}$  in an energy-dependent process from the cytosol to the extracellular space and into intracellular compartments such as the vacuole and the endoplasmic reticulum (ER). As a result, the  $[\text{Ca}^{2+}]$  in the apoplast and intracellular stores is up to 20,000 times higher than in the cytosol. The combination of this concentration gradient and a proton-based electrochemical gradient over cellular membranes [8] is the driving force for the stimulus-dependent influx of  $\text{Ca}^{2+}$  into the cytosol [7].

Genome-wide comparisons revealed large differences between families for channel candidates mediating  $\text{Ca}^{2+}$  import in plants and mammals [8-10]. Genes encoding obvious orthologs of some major classes of mammalian channels are lacking in plants (ryanodine receptor channels,  $\text{InsP}_3$ -activated receptor channels and voltage-gated channels), while the family of cyclic nucleotide-gated channels (CNGCs) is expanded in plants. Even though sequence-related genes are missing, electrophysiological experiments indicated the presence of voltage-gated  $\text{Ca}^{2+}$  channels in plant organelles and cells [11, 12]. Apparently, a unique and sequence-diverged set of proteins contributes to the control of  $\text{Ca}^{2+}$  influx into plant cells.

CNGCs comprise a family of twenty ligand-gated ion channels in *Arabidopsis thaliana* [8]. CNGCs are gated by cyclic nucleotides (cNTPs) [13, 14]. Several CNGCs have been functionally linked to plant immunity. For example, the *defense no death* (*dnd*) class of mutants has a reduced ability to initiate a hypersensitive response (HR) to avirulent bacteria. *DND1* and *DND2* encode two CNGC paralogs (CNGC2 and CNGC4) [15, 16]. In addition to the altered cell death phenotype, *cngc2* knockout plants show an altered  $\text{Ca}^{2+}$  signature in response to the bacterial MAMP lipopolysaccharide (LPS) [13].

The second major class of putative ligand-gated  $\text{Ca}^{2+}$ -permeable channels in plants comprises the ionotropic glutamate receptor-like channels (iGluRs). They function as heterotetramers and are best known for their role in neurotransmission in vertebrates [17]. iGluR-like channels possess a large N-terminal extracellular domain, three transmembrane regions, a hydrophobic loop defining the pore region and a cytosolic C-terminal domain. Especially the pore region of iGluR-like channels differs strongly between plants and animals [18, 19]. However, ion pore transplantation experiments have shown that the pores of two *Arabidopsis* iGluRs (AtiGluR1.1 and AtiGluR1.4) are permeable to  $\text{Ca}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$  [18]. Twenty iGluRs are encoded in the *Arabidopsis* genome [9, 10, 19]. They are involved in various processes ranging from biotic and abiotic stress responses [20] to pollen tube

iGluRs mediate MAMP-triggered calcium influx in *Arabidopsis*

growth [21] and hypocotyl elongation [22]. Recently,  $\text{Ca}^{2+}$  influx and NO production triggered by the proteinaceous oomycete MAMP cryptogein were also found to involve iGluR-like channels [23].

Here we used a transgenic *Arabidopsis thaliana* line expressing the aequorin  $\text{Ca}^{2+}$  biosensor to investigate intracellular  $\text{Ca}^{2+}$  signatures in response to three different, well-characterized MAMPs (flg22, elf18 and chitin). We conducted pharmacological interference with the MAMP-triggered  $\text{Ca}^{2+}$  influx using a comprehensive set of compounds known to impede various classes of  $\text{Ca}^{2+}$  channels. Besides the actual  $\text{Ca}^{2+}$  signatures, we used MAMP-induced downstream responses such as MAPK activation and accumulation of defense gene transcripts as additional readouts for  $\text{Ca}^{2+}$  channel modulation. Our findings support a crucial role of iGluR-like channels in the generation of MAMP-triggered  $\text{Ca}^{2+}$  signatures and provide evidence for a critical role for MAMP-induced  $\text{Ca}^{2+}$  signaling in defense gene activation.

## Experimental

### Plant growth

*Arabidopsis* Col-0 seeds or Col-0 35S::aequorin seeds [24] were surface-sterilized once with 70% ethanol and subsequently twice with 100% ethanol before drying. Dry seeds were transferred to a 96-well plate (Perkin-Elmer, Waltham, USA; 1-2 seeds per well) containing 100  $\mu\text{l}$  1x MS including vitamins (DUCHEFA, Haarlem, the Netherlands) with 0,25% sucrose. The seedlings were grown for 11-12 days with 16 hours light (21  $^{\circ}\text{C}$ ), 8 hours dark (19  $^{\circ}\text{C}$ ) before further study.

### Chemicals

Unless stated otherwise, chemicals were obtained from SIGMA (Munich, Germany) or Tocris Biosciences (Ellisville, USA). Stock solutions were prepared in ddH<sub>2</sub>O except for CNQX, U73122 and nifedipine, which were dissolved in DMSO. The influence of the solvent DMSO in the final concentration after chemical dilution was tested as an additional control.

### $\text{Ca}^{2+}$ measurements using the aequorin biosensor

After 11-12 days the growth medium was removed from the aequorin expressing seedlings and replaced by 100  $\mu\text{l}$  ddH<sub>2</sub>O containing 10  $\mu\text{M}$  coelenterazine (BIOSYNTH, Staad, Switzerland), diluted from a 5 mM stock solution in methanol, and incubated overnight in the dark. The next day, the medium was replaced by 100  $\mu\text{l}$  ddH<sub>2</sub>O and the seedlings were incubated for 30 min in the dark. For the compound treatments, 10-times concentrated stocks in water were prepared and added to the coelenterazine-loaded seedlings. The seedlings were preincubated for one hour before starting the measurements. Elf18 and flg22 were present as 10 or 1 mM stock solutions in water stored at -80  $^{\circ}\text{C}$  and used freshly. Chitin solution was prepared by intermittently grinding (for 15-20 minutes) 10 mg crab or shrimp shell (SIGMA, Munich, Germany) powder in 1 ml ddH<sub>2</sub>O with a pestle and subsequent vortexing and heating the solution to 60  $^{\circ}\text{C}$ . Undissolved particles were removed by centrifugation (15 minutes, 13000 rpm) and the clear supernatant was used for the experiments. The measurements were performed in a 96-well Centro LB960 luminometer system (Berthold Technologies, Bad Wildbad, Germany). Half a plate was measured in one go. Luminescence from one single well was detected for 0,25 seconds and each well was measured every 30 seconds. After 2 minutes (4 measurements), 5-times concentrated MAMP was automatically added to a final concentration of 1  $\mu\text{M}$  flg22, 1  $\mu\text{M}$  elf18 or 0,1 mg/ml chitin. After addition, the luminescence was followed for an additional 40 minutes. The second half of the plate was run in the same manner. Finally, for calculation of  $\text{Ca}^{2+}$  concentrations, 100  $\mu\text{l}$  2 M  $\text{CaCl}_2$  in 20% ethanol was added to each well using the injectors of the system and the luminescence was measured for 0,25s per well with one measurement per well every 63 seconds. The luminescence was followed for 30 minutes.  $\text{Ca}^{2+}$  concentrations were calculated according to Rentel and Knight (2004)[25].

Per treatment 4 to 6 individual wells were averaged.  $\text{Ca}^{2+}$  transients were compared between treatments within one experiment unless stated otherwise and at least three biological replicates per compound were performed. Unless stated otherwise, the data were normalized to background  $\text{Ca}^{2+}$  levels and represented as a  $\Delta[\text{Ca}^{2+}]$  value to compensate for background variations between measurements. Owing to the low molecular mass of aequorin (22 kDa) and its presumed passive



iGluRs mediate MAMP-triggered calcium influx in *Arabidopsis*

transfer into the nucleus, we expect the *Arabidopsis* reporter line to monitor the combined cytoplasmic and nuclear (here referred to as intracellular)  $\text{Ca}^{2+}$  pool. Statistical significance of differences was evaluated by comparing average calcium responses of biological replicates of treated samples with non-treated control samples using a two-sided Student's t-test with unequal variances.

**MAPK assays**

The MAPK assays were performed essentially as previously described [26].

**Quantitative RT-PCR**

Preparation, pretreatment and MAMP addition to the seedlings was identical as described for the MAPK assays. Time point 0 was harvested before MAMP addition and 30 and 60 minutes after MAMP addition. Total RNA was isolated using the RNeasy Plant Kit (QIAGEN, Hilden, Germany). cDNA was synthesized with 5  $\mu\text{g}$  total RNA, oligo dT primers and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturers protocol. Transcript abundance was quantified using the IQ5 real-time PCR thermocycler (Bio-Rad, Munich, Germany). The expression of the genes of interest was normalized to *At4g26410* (for primers used see [27]) and to the relative transcript abundance in non-treated Col-0 control samples as calculated according to the comparative cycle threshold ( $\Delta\Delta\text{Ct}$ ) method [28]. Transcript abundance of a MAPK dependent gene, *FRK1* and a CDPK dependent gene, *PHI-1*, were tested (for primers used see [29]). Three biological replicates with three technical replicates per sample were performed per treatment.

**Results****MAMP-triggered  $\text{Ca}^{2+}$  influx from the apoplast**

We used the luminescent aequorin  $\text{Ca}^{2+}$  sensor system [24] to record whole seedling intracellular  $\text{Ca}^{2+}$  transients in response to MAMPs. Owing to previous in-depth characterization and the identification of the cognate PRRs we focused on responses towards the bacterial peptide elicitors flg22 and elf18 [2, 3] as well as chitin, a carbohydrate polymer that represents the major constituent of fungal cell walls [30]. To narrow down candidate protein classes involved in regulating MAMP-triggered  $\text{Ca}^{2+}$  signatures in *Arabidopsis*, a range of chemicals were chosen that are known to target components controlling  $\text{Ca}^{2+}$  in- and efflux in plant and animal systems. Due to potential plant off-targets, presumed differences in bioavailability, compound metabolism and dissimilarities in the molecular structure of plant and animal targets we generally tested multiple chemicals affecting the same molecule class.

To investigate the source of the initial intracellular [ $\text{Ca}^{2+}$ ] rise, the general  $\text{Ca}^{2+}$  channel inhibitor Lanthanum chloride ( $\text{LaCl}_3$ ) and a chelator of extracellular bivalent cations, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), were applied. Consistent with previous reports [6, 31] flg22 and elf18 induced a biphasic  $\text{Ca}^{2+}$  response with a maximum [ $\text{Ca}^{2+}$ ] at about three minutes after MAMP addition and a recovery phase of ca. 20-30 minutes (Figures 1A and 1B). Chitin induced a monophasic  $\text{Ca}^{2+}$  transient (Figure 1C). Preincubation of the seedlings with either  $\text{LaCl}_3$  or EGTA fully inhibited the  $\text{Ca}^{2+}$  transients induced by all three MAMPs, suggesting the main  $\text{Ca}^{2+}$  source for the MAMP-triggered spikes is the apoplast (Figures 1, A-D, Supplemental Figures S1A and B and Table 1). In the presence of EGTA, a continuous rise in intracellular  $\text{Ca}^{2+}$  levels was observed, independent of the presence or absence of MAMPs (Figure 1D, Supplemental Figure S1A and B).

**An ambiguous role for internal  $\text{Ca}^{2+}$  stores in MAMP-triggered  $\text{Ca}^{2+}$  influx**

To further validate the conclusion that the major source of MAMP-induced  $\text{Ca}^{2+}$  is the apoplast we studied the role of phospholipase C (PLC)-dependent release of  $\text{Ca}^{2+}$  from intracellular stores using the PLC inhibitors neomycin and U73122 [32, 33]. Both inhibitors led to increased steady state  $\text{Ca}^{2+}$  levels (Figure 2A, Supplemental Figure S2C and Table 1). U73122 did not alter  $\Delta[\text{Ca}^{2+}]$  for the tested MAMPs, but changed the shape of the response curves of elf18 and chitin (Figure 2B, Supplemental Figures S2A and B), which suggests a minor contribution for PLC-controlled  $\text{Ca}^{2+}$  release in response to these MAMPs. Neomycin did not alter the chitin-induced  $\Delta[\text{Ca}^{2+}]$  (Supplemental Figure S2F), but caused a slight reduction in the flg22- and elf18-induced  $\Delta[\text{Ca}^{2+}]$  (Supplemental Figures S2C-E). In

iGluRs mediate MAMP-triggered calcium influx in *Arabidopsis*

conclusion, PLC-mediated  $\text{Ca}^{2+}$  release has a minor, if any, contribution to the MAMP-triggered  $\Delta[\text{Ca}^{2+}]$ .

A MAMP-triggered primary apoplastic  $\text{Ca}^{2+}$  influx does not exclude the possibility of secondary  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) from internal stores. Ruthenium Red (RR) inhibits CICR from internal stores in plant and animal systems [34], but also inhibits certain channels at the plasma membrane [35, 36]. Like inhibition of the PLC pathway, addition of RR resulted in increased steady state  $[\text{Ca}^{2+}]$  (Supplemental Figure S2G and Table 1). In addition, the chitin- and elf18-triggered  $\Delta[\text{Ca}^{2+}]$  were strongly reduced compared to the uninhibited situation (Figures 2C and D), while the flg22-induced  $\Delta[\text{Ca}^{2+}]$  was largely unaffected (Supplemental Figures S2G and H). This suggests either a significant contribution of CICR from intracellular stores or, alternatively, a role for specific RR-targeted plasma membrane-localized channels in the chitin and elf18 responses. Like RR, tetracaine directly targets mammalian ryanodine receptors and blocks CICR from intracellular stores [34]. Tetracaine induced a higher  $\Delta[\text{Ca}^{2+}]$  for all three MAMPs and interfered with the recovery phase, visible as irregular spikes during the decline of the  $\text{Ca}^{2+}$  response (Figure 2E, Supplemental Figures S2I-J and Table 1). Unlike for the other intracellular store channel inhibitors, in the case of tetracaine no increased steady state  $[\text{Ca}^{2+}]$  was observed (not shown). In sum, the compounds targeting internal  $\text{Ca}^{2+}$  stores yielded ambiguous data. A role for PLC-dependent  $\text{Ca}^{2+}$  release and/or CICR in response to MAMP treatment can thus not be ruled out.

#### **Inhibitors of voltage- and nucleotide-gated channels do not affect MAMP-triggered $\text{Ca}^{2+}$ influx**

To further delimit the channels involved in  $\text{Ca}^{2+}$  import from extracellular stores, a set of inhibitors targeting animal type voltage-gated  $\text{Ca}^{2+}$  channels (VGCC) were tested. Although plants lack obvious candidate genes coding for VGCCs, electrophysiological data support their existence in plant organelles and cells [11, 12]. None of the three L-type VGCC inhibitors, verapamil, diltiazem and nifedipine, altered the  $\Delta[\text{Ca}^{2+}]$  after MAMP stimulus (Figure 2F and Supplemental Figures S3A-H and Table 1). Nifedipine treatment resulted in a delayed recovery in the case of all three MAMPs (Figure 2F and Supplemental Figures S3A-B and Table 1). Verapamil-treated seedlings occasionally showed random spiking after the MAMP stimulus (Supplemental Figures S3C-E and Table 1). Together, these results suggest that the sensing of flg22, elf18 or chitin does not activate  $\text{Ca}^{2+}$  influx through VGCCs.

Plant genomes encode two major classes of putative  $\text{Ca}^{2+}$  channels, CNGCs and iGluRs. Two compounds were tested that interfere with cNTP biosynthesis and therefore indirectly inhibit CNGC function, alloxan and dideoxyadenosine (DDA). Neither incubation of seedlings with alloxan nor treatment with DDA caused an alteration of the MAMP-induced  $\Delta[\text{Ca}^{2+}]$  for flg22 (Figures 3A and B, Supplemental Figure S5C and Table 1). For elf18 and chitin the  $\Delta[\text{Ca}^{2+}]$  was slightly lower in the presence of alloxan or DDA (Supplemental Figures S4A-D, Supplemental Figures S5F and I and Table 1) DDA pretreatment resulted in a delayed recovery of cellular  $\text{Ca}^{2+}$  levels after MAMP stimulation (Figure 3B, Supplemental Figures S4C and D and Table 1). These results suggest that, dissimilar to lipopolysaccharides (LPS) [13] cyclic nucleotide biosynthesis and thus likely also CNGCs do not make a major contribution to the flg22-, elf18- or chitin-induced  $\text{Ca}^{2+}$  influx.

#### **Inhibition of iGluR-like channels affects MAMP-triggered $\text{Ca}^{2+}$ influx**

The second major class of putative  $\text{Ca}^{2+}$  channels in plants comprises the iGluR-like channels, which include 20 members in *Arabidopsis* [9, 10, 19]. We employed various inhibitors directed against NMDA- and non-NMDA-like mammalian iGluRs. AP-5 and AP-7, competitive antagonists of the glutamate binding site [37] and kynurenic acid, a competitive antagonist of the glycine binding site [38], caused a strong inhibition of the MAMP-triggered  $\Delta[\text{Ca}^{2+}]$ . For all three MAMPs, the reduction induced by both AP-5 and kynurenic acid was statistically significant ( $p < 0.05$ ). Treatment with these inhibitors also led to increased steady state  $[\text{Ca}^{2+}]$  (Figures 4A-C, Supplemental Figures S4E-H and Table 1). The observation that increased cellular  $\text{Ca}^{2+}$  concentrations in the presence of U73122 still allowed a normal flg22-, elf18- and chitin-induced  $\Delta[\text{Ca}^{2+}]$  (Figures 2A and B, Supplemental Figures S2A and B) suggests that elevated steady state  $\text{Ca}^{2+}$  levels do not per se impede a MAMP-triggered  $\text{Ca}^{2+}$  transient. Other iGluR channel inhibitors tested targeting either the channel domain of NMDA-like channels (MK-801) [37] or non-NMDA iGluR channels (DNQX, CNQX) [39] did not inhibit MAMP-triggered  $\Delta[\text{Ca}^{2+}]$  (Supplemental Figure S6 and Table 1). Together, these data provide first

iGluRs mediate MAMP-triggered calcium influx in *Arabidopsis*

evidence for a potential role of iGluR-like channels in the generation of the flg22-, elf18- and chitin-triggered  $\text{Ca}^{2+}$  transient.

To further analyze the potential role of iGluRs in MAMP-induced  $\text{Ca}^{2+}$  influx, we exploited the phenomenon of iGluR desensitization [40]. If iGluRs were the channels mediating MAMP-triggered  $\text{Ca}^{2+}$  influx, then agonistic amino acids such as L-glutamate and L-aspartate should desensitize the channels for subsequent MAMP treatment, while other amino acids should have either less or no effect. We thus monitored the MAMP-triggered  $\text{Ca}^{2+}$  response after preincubation of the aequorin-expressing seedlings with ten different amino acids that differ largely in their physiochemical properties (Figure 4F). Pretreatment of seedlings with both L-glutamate and L-aspartate resulted in a statistically significant reduction ( $p < 0,05$ ) of the  $\Delta[\text{Ca}^{2+}]$  after flg22 stimulation (Figures 4D-E, Supplemental Figures S4I-J and Table 1). In addition, reminiscent of treatment with the inhibitors AP-5, AP-7 and kynurenic acid, basal cellular  $[\text{Ca}^{2+}]$  levels were elevated to 125%-158% of the control following application of these two negatively charged amino acids (Figure 4D and F and Table 1). Other amino acids did neither reduce the MAMP-induced  $\Delta[\text{Ca}^{2+}]$  nor affect basal cellular  $[\text{Ca}^{2+}]$  (Figure 4F).

We then tested a range of L-glutamate concentrations for desensitization of the flg22-mediated  $\text{Ca}^{2+}$  transient and established a dose-response curve (Figure 4G). We found a concentration-dependent reduction of  $\Delta[\text{Ca}^{2+}]$  and a reciprocal concentration-dependent increase in steady state  $\text{Ca}^{2+}$  levels. From a concentration of 0,2 mM L-glutamate onwards a reduction of  $\Delta[\text{Ca}^{2+}]$  and an increase in basal cellular  $[\text{Ca}^{2+}]$  was observed. Pretreatment with concentrations above 0,6 mM L-glutamate resulted in maximal inhibition of the flg22-triggered  $\text{Ca}^{2+}$  transient, indicating saturation of the system (Figure 4G). The concentration range where L-glutamate desensitized MAMP-induced  $\text{Ca}^{2+}$  influx is in agreement with the reported  $K_m$  values for plant iGluR-like channels, which were found to be between 0,2 and 0,5 mM [41], similar to reported apoplasmic amino acid concentrations (0,3-1,3 mM) [42, 43]. In sum, the results of desensitization experiments further support a role of iGluRs in MAMP-triggered  $\text{Ca}^{2+}$  influx.

**iGluR inhibitors and L-glutamate modulate MAMP-induced MAPK activation**

To expand the analysis of cellular responses induced by compounds that affect iGluR activity, we inspected other early MAMP-triggered events. Upon biotic stress specific MAPK signaling cascades are activated, which control additional cellular responses and defense gene expression [44]. We examined MAMP-induced MAPK activation in the presence or absence of L-glutamate or the iGluR inhibitors kynurenic acid or CNQX. Application of L-glutamate as a stimulus to seedlings resulted in low levels of MAPK activation, visible as three additional bands (MPK6, MPK3 and MPK4) appearing at five minutes after addition of L-glutamate or the MAMP stimulus (Figure 5A). Preincubation with either L-glutamate or kynurenic acid for one hour resulted in low levels of MAPK activation. Subsequent addition of more L-glutamate still transiently increased activated MAPK levels. Preincubation of the seedlings with CNQX, which did not affect the MAMP-triggered  $\text{Ca}^{2+}$  signature, completely abolished MAPK activation after L-glutamate addition (Figure 5A).

Flg22, elf18 and chitin induced strong MAPK activation within 5 minutes after MAMP addition (Figures 5B and C and Supplemental Figure S7). In the presence of L-glutamate, kynurenic acid or CNQX, the flg22 and elf18 response was only affected to a minor extent. The presence of L-glutamate or kynurenic acid often delayed MAPK activation, with full activation not before 15 minutes (Supplemental Figure S7). In contrast, all three compounds markedly reduced the chitin-triggered activation of the MAPK(K/KK) cascade (Figure 5B).

Recently, Boudsocq and co-workers [29] showed that flg22-induced MAPK activation in *Arabidopsis* protoplasts does not depend, but is enhanced by  $\text{Ca}^{2+}$  influx. To corroborate this observation in intact *Arabidopsis* seedlings and to compare it with the glutamate-dependent inhibition of MAPK activation, we performed MAPK assays in the presence of either EGTA or  $\text{LaCl}_3$ . In the case of flg22, full MAPK activation was delayed by both EGTA and  $\text{LaCl}_3$  (Supplemental Figure S7B). In the case of elf18 and chitin, EGTA reduced and  $\text{LaCl}_3$  delayed MAPK activation (Figure 5C and Supplemental Figure S7D). In conclusion, MAMP-triggered MAPK signaling requires  $\text{Ca}^{2+}$  influx for full and timely activation. Modulation of MAMP-triggered  $\text{Ca}^{2+}$  influx by iGluR inhibitors or glutamate desensitization affected MAPK activation in a similar manner as full inhibition (Figures 1A-D) of apoplasmic  $\text{Ca}^{2+}$  influx by the potent non-selective chemicals  $\text{LaCl}_3$  and EGTA.



### Cooperative regulation of Ca<sup>2+</sup> influx by CNGC and iGluR-like channels?

Modulating glutamate-controlled Ca<sup>2+</sup> influx did largely but not fully abolish the MAMP-triggered Ca<sup>2+</sup> transient (Figure 4; Supplemental Figures S4 and S5). Therefore we wondered if cNTP-regulated Ca<sup>2+</sup> import was responsible for the remaining influx of Ca<sup>2+</sup> and possibly a positive feedback loop exists between both channel types. Plants were co-treated with both kynurenic acid/L-glutamate and alloxan/dideoxyadenosine before MAMP addition and both Ca<sup>2+</sup> levels and MAPK activation were followed. For elf18, but not for flg22 and chitin, the combined incubations gave a stronger reduction in  $\Delta[\text{Ca}^{2+}]$  than each of the single compounds (compare Figure 6A with Supplemental Figures S8A and C, Table 1). As discussed above, L-glutamate pretreatment reduced (chitin) or delayed (flg22 and elf18) MAPK activation. Alloxan pretreatment did not influence the intensity of the two major MAPK bands (MPK6 and MPK3), but reduced the intensity of the MAPK band with the highest electrophoretic mobility, which likely represents MPK4 (Figure 6B and Supplemental Figures S8B and D). The co-inhibition of cNTP- and iGluR-mediated Ca<sup>2+</sup> influx resulted in a combination of both effects; the major MAPK bands representing MPK3 and 6 showed a delayed activation (elf18) or reduced intensity (chitin) and nearly no activation of MPK4 could be detected anymore (Figure 6B and Supplemental Figures S8B, D and F). In conclusion, we did not find evidence for a strong cooperative effect of iGluR-like channels and CNGCs in MAMP-triggered Ca<sup>2+</sup> influx.

### Glutamate signaling differentially modulates MAPK- and CDPK-dependent defense gene transcript levels in response to MAMPs

To investigate how the modulation of iGluR-like channels impacts on MAMP-triggered transcriptional activation of defense genes, we studied transcript accumulation of each a MAPK- and a CDPK-dependent gene (*FLG22-INDUCED-RECEPTOR-LIKE-KINASE1*, *FRK1*, and *PHOSPHATE-INDUCED1*, *PHI-1*, respectively) [29] using quantitative RT-PCR. *FRK1* transcript accumulation was rapidly (within 30 minutes) induced by each of the three MAMPs (Figure 7A). Application of either L-glutamate or kynurenic acid reduced basal *FRK1* expression levels and drastically impaired MAMP-dependent accumulation of *FRK1* transcripts (Figure 7A). *PHI-1* transcript levels were strongly and transiently induced by flg22 and moderately by elf18 or chitin (Figure 7B). L-glutamate and kynurenic acid each increased basal *PHI-1* expression levels and blocked an additional increase in transcript accumulation upon MAMP treatment. Thus, modulation of MAMP-triggered Ca<sup>2+</sup> influx by L-glutamate or kynurenic acid inhibits transcriptional up-regulation of genes downstream of the MAPK (*FRK1*) and CDPK (*PHI-1*) signaling cascades.

## Discussion

We tested a comprehensive set of compounds known to target various components regulating Ca<sup>2+</sup> signaling for their influence on MAMP-induced Ca<sup>2+</sup> influx in *Arabidopsis* seedlings. Pharmacological approaches bear the risk of unspecific off-targets, but offer the advantage to overcome potential redundancy in target function and allow transient inhibition with minimal pleiotropic effects. We opted for a pharmacological approach to surmount the presumed genetic redundancy of plant Ca<sup>2+</sup> channels and the expected pleiotropic phenotypes of respective mutants. We aimed to minimize the impact of off-targets on our data by applying multiple inhibitors per target molecule class in most cases.

### Inhibition of iGluR-like channels affects MAMP-triggered Ca<sup>2+</sup> influx

Flg22 and elf18 induced biphasic Ca<sup>2+</sup> transients that were very similar in shape and timing, while chitin caused a monophasic Ca<sup>2+</sup> transient (e.g. Figure 1A and C). The presence of two separate maxima in the case of flg22 and elf18 suggests either the involvement of different channel types, different subcellular Ca<sup>2+</sup>-stores or a differential responsiveness between cell types. The aequorin reporter system used in this study does not allow the detection of changes in [Ca<sup>2+</sup>] at the (sub)cellular level. Therefore, other reporters like the yellowameleon FRET sensor are desirable for future analyses with improved spatial resolution [46, 47].

iGluRs mediate MAMP-triggered calcium influx in *Arabidopsis*

LaCl<sub>3</sub>-mediated inhibition of plasma membrane-resident ion channels and chelating external Ca<sup>2+</sup> via EGTA treatment revealed that the primary Ca<sup>2+</sup> pool for both types of MAMP-triggered Ca<sup>2+</sup>-signatures is the apoplast (Figures 1A-D and Supplemental Figures S1A-C). This confirms and extends findings of a previous study [45], where LaCl<sub>3</sub> was used to demonstrate that flg22-induced Ca<sup>2+</sup> influx is needed for membrane depolarization in mesophyll cells. Furthermore, both inhibition of Ca<sup>2+</sup> influx by LaCl<sub>3</sub> and chelating apoplastic Ca<sup>2+</sup> were shown to prevent CDPK activation and to reduce MAPK activation in response to the peptide elicitors flg22 and elf18 in *Arabidopsis* protoplasts [29]. These findings suggest a role for Ca<sup>2+</sup> signaling in modulating downstream responses during MAMP-triggered immunity.

Interference with InsP<sub>3</sub> or CICR resulted in increased steady state Ca<sup>2+</sup> levels for three of the inhibitors tested (U73122, Neomycin and RR). Notably, although basal Ca<sup>2+</sup> levels were increased, a normal Ca<sup>2+</sup> transient was induced (Figures 2A-B). In addition, RR reduced the chitin- and elf18-induced Ca<sup>2+</sup> transients, possibly due its inhibitory effect on plasma membrane-resident ion channels [35, 36]. The fourth compound, tetracaine, caused a slightly enhanced Ca<sup>2+</sup> transient, but did not alter basal Ca<sup>2+</sup> levels.

Inhibition of VGCCs did not change the intensity of MAMP-triggered Ca<sup>2+</sup> transients, but for two inhibitors (verapamil and nifedipine) delayed the recovery to basal cytosolic Ca<sup>2+</sup> levels (Figure 2F and Supplemental Figure S3). VGCC inhibitors have documented effects in plants [35, 48]. However, all three L-type channel inhibitors have also other reported targets than L-type Ca<sup>2+</sup> channels, including sodium channels and membrane receptors (<https://www.ebi.ac.uk/chembl/db/>). The prolonged Ca<sup>2+</sup> signature might thus be due to interference with other ion channel types and a resulting altered recovery of the membrane potential or ion gradients including Ca<sup>2+</sup>.

Inhibitors of CNGCs previously shown to inhibit CNGC-mediated responses *in planta* [13], DDA and alloxan, caused only a slight reduction in the MAMP-triggered Ca<sup>2+</sup> transients. Therefore in contrast to LPS [13] and the Danger-Associated Molecular Pattern (DAMP) AtPep3 [14], cNTPs and thus likely CNGCs do not control the flg22-, elf18- and chitin-induced Ca<sup>2+</sup> transient. *cngc2* mutants lose the LPS-induced Ca<sup>2+</sup> increase [13], but show normal flg22-triggered membrane depolarization [45]. It appears that the MAMPs used in the present study activate a different set of ion channels than LPS does. The proposed discrepancy between the channels involved is in line with the different gene sets activated by the two groups of elicitors [29]. Surprisingly, the flg22-induced Ca<sup>2+</sup> transient is attenuated in a *Atppepr1* mutant background [14], which suggests crosstalk at the level of Ca<sup>2+</sup> influx between the AtPep and flg22/elf18/chitin pathways independent of cNTP generation. The delayed recovery of the MAMP-induced cytosolic rise in Ca<sup>2+</sup> levels in the presence of DDA suggests a role for cNTPs in controlling Ca<sup>2+</sup> efflux.

Of the six iGluR inhibitors tested, AP-5, AP-7 and kynurenic acid resulted in a strong reduction in the MAMP-induced Δ[Ca<sup>2+</sup>] and all three led to an increase in cytosolic [Ca<sup>2+</sup>] (Figures 4A-C, Supplemental Figures S4-6 and Table 1 and 2). MK-801, CNQX and DNQX pretreatment did not alter flg22-, elf18- or chitin-induced Ca<sup>2+</sup> fluxes (Supplemental Figure S6). AP-5, CNQX and DNQX have been previously shown to affect iGluR-mediated processes and Ca<sup>2+</sup> currents in pollen tube growth (AP-5, CNQX, DNQX) [21], hypocotyl elongation (DNQX) [22], glutamate-induced Ca<sup>2+</sup> transients in mesophyll cells (CNQX, DNQX) [20] and responses to Al<sup>3+</sup>-induced Ca<sup>2+</sup> transients in roots (AP-5) [48]. Most recently, AP-5 was found to strongly inhibit Ca<sup>2+</sup> influx induced by the peptide elicitor cryptogein in tobacco cell culture, while MK-801 and DNQX were significantly less effective in these experiments [23]. Based on these published findings, a subset of the iGluR inhibitors can efficiently modulate both NMDA- and non-NMDA-like iGluR channels. In our hands, DNQX, CNQX and MK-801 did not result in inhibition of MAMP-induced Ca<sup>2+</sup> influx (Supplemental Figure S6). Possibly these compounds were unstable in our experimental conditions or were unable to penetrate the seedling tissue well enough to compete with glutamate for binding sites. In pollen tubes, AP-5 had a stronger influence on Ca<sup>2+</sup> currents than CNQX and DNQX, suggesting that higher apoplastic concentrations of the latter compounds are needed [21], which might not have been achieved in our experimental set-up. Alternatively, various plant iGluR channels or iGluR channel complexes may differ in their sensitivity towards the inhibitors. The channel domain of some plant iGluR-like channels are permeable to Ca<sup>2+</sup>, Na<sup>+</sup> and K<sup>+</sup> [18], but they differ in conserved amino acids controlling channel selectivity from animal iGluRs [18, 19], which might explain the inactivity of MK-801 in our experiments. Interestingly, inhibitor treatment and de-sensitization of the MAMP responses with L-

iGluRs mediate MAMP-triggered calcium influx in *Arabidopsis*

glutamate or L-aspartate resulted in similar changes of the  $\text{Ca}^{2+}$  response and increased steady state  $[\text{Ca}^{2+}]$  (Figure 4, Table 1 and 2). The increased steady state  $\text{Ca}^{2+}$  levels in the presence of both inhibitors and agonists can be best rationalized by either an unexpected partial agonistic function of the presumed inhibitors or by hyper-compensation of interference with iGluR-like channel function via other  $\text{Ca}^{2+}$  channel types.

Inhibition of iGluR-like channel does not fully abolish the  $\text{Ca}^{2+}$  spike, which suggests either partial inhibition of these channels or the involvement of a second channel class in the response. A second channel class acting in the flg22- and elf18-mediated  $\text{Ca}^{2+}$  influx would explain the biphasic shape of the  $\text{Ca}^{2+}$  response for these MAMPs. However, chitin-induced fluxes are altered in a similar manner, which argues for an alternative explanation of the phenomenon. Our experiments suggest the potential second class of channels is not cNTP-activated, since co-treatment with inhibitors of adenylate cyclases and iGluRs did not fully block  $\text{Ca}^{2+}$  influx (Figure 6A).

We exploited the phenomenon of iGluR channel desensitization by analyzing the MAMP response after pretreatment with various amino acids. In our experiments, flg22-, elf18- and chitin-induced responses were only desensitized by the negatively charged L-glutamate and L-aspartate (Figures 4D-G, Supplemental Figures S4I and J). In a previous study certain combinations of amino acids were tested for cross-desensitization and the dependence on the presence of AtGLR3.3 and AtGLR3.4 [40]. The results of this work fit a model where various quaternary complexes of iGluR-like channels co-exist that vary in their sensitivity to different amino acids. Taking this model into account, the iGluR complex(es) activated by MAMPs only seem(s) to contain subunits sensitive to both L-glutamate and L-aspartate. Comparative modeling of the glutamate binding site of plant iGluR-like channels suggested that only AtGLR1.1 can bind glutamate, while the other family members bind glycine [22]. This suggests that the iGluR complex responsible for MAMP-triggered  $\text{Ca}^{2+}$  influx would have to contain AtGLR1.1. However, modeling studies alone are insufficient to exclude binding of other amino acids [49]. Furthermore, L-glutamate or L-aspartate might not directly bind the receptor, but induce the release of another compound that modulates iGluR-like channel activity [49].

### iGluR-mediated $\text{Ca}^{2+}$ influx controls defense gene expression

Reduction of MAMP-triggered  $\text{Ca}^{2+}$  influx fully inhibited expression of a MAPK-dependent gene (*FRK1*) and increased steady state levels of a CDPK-dependent gene (*PHI-1*) (Figure 7) [29]. Given the only modest effect of L-glutamate and kynurenic acid on MAPK activation (Figure 5 and Supplemental Figure S7) the strong impact on MAPK-dependent *FRK1* expression was surprising. This result suggests that either the signal transduction cascade leading from  $\text{Ca}^{2+}$  influx to the activation of *FRK1* transcript accumulation relies only partially on MAPK activation, or that a moderate impact on MAPK activity can have a profound effect on downstream gene expression. The enhanced steady state *PHI-1* transcript levels seen upon treatment with L-glutamate or kynurenic acid are possibly a consequence of the enhanced intracellular steady state  $\text{Ca}^{2+}$  levels caused by these compounds, which may lead to constitutive low level activation of CDPKs. Notably, a further MAMP-induced increase in *PHI-1* transcript levels was also blocked by L-glutamate and kynurenic acid. In sum, these data indicate a crucial role for cellular signaling events upon MAMP perception that rely on iGluR-mediated  $\text{Ca}^{2+}$  influx. It remains to be seen to what extent the reduction in defense gene activation translates into a compromised immune response. In this context it would be highly desirable to examine the effect of interference with  $\text{Ca}^{2+}$  signaling during authentic plant-microbe interactions. However, the dual effect of compounds on both the host and the parasite combined with the resulting complication in interpreting the data from such experiments emphasize the limitations of pharmacological approaches.

### A model for iGluR-mediated $\text{Ca}^{2+}$ influx

Based on our data we propose the following model for MAMP-triggered  $\text{Ca}^{2+}$  influx (Figure 8): Following MAMP perception by cognate PRRs, glutamate-dependent opening of iGluR-like channels and  $\text{Ca}^{2+}$  influx from the apoplast into the cytoplasm is activated by yet unknown mechanisms. This may involve vesicle-mediated secretion of L-glutamate into the apoplast [23]. Elevated cytoplasmic  $\text{Ca}^{2+}$  levels translate via CDPK- and MAPK-dependent pathways into defense gene activation. It remains unclear how  $\text{Ca}^{2+}$  influx via iGluRs is terminated. One possibility is a negative feedback loop that involves  $\text{Ca}^{2+}$ -dependent calmodulin (CaM) binding to hypothetical CaM binding domain(s) of

iGluR-like channels. As a next step, reverse genetic approaches are required to further substantiate the findings of the present study at a different experimental level. In our hands the analysis of a subset of iGluR single mutants did not yet result in the identification of mutants that exhibit altered MAMP-induced  $\text{Ca}^{2+}$  signatures. These preliminary data indeed argue for redundant iGluR functions in plant innate immunity.

#### **Acknowledgements**

We thank Justine Lorek for providing protocols and primer sequences for qRT-PCR and MAPK assays. We are grateful to the lab of Tina Romeis for performing CDPK activity assays. We acknowledge Dierk Scheel and Mark Knight for kindly providing seeds of the 35S-aequorin line. We apologize to authors whose work could not be cited due to space constraints.

#### **Funding**

Work in the lab of RP is supported by grants from the Max-Planck society and the Deutsche Forschungsgemeinschaft (DFG; SFB670).



## References

- 1 Boller, T. and Felix, G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* **60**, 379-406
- 2 Gómez-Gómez, L. and Boller, T. (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol. Cell* **5**, 1003-1011
- 3 Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D., Boller, T. and Felix, G. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* **125**, 749-760
- 4 Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H. and Shibuya, N. (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 19613-19618
- 5 Lecourieux, D., Ranjeva, R. and Pugin, A. (2006) Calcium in plant defence-signalling pathways. *New Phytol.* **171**, 249-269
- 6 Aslam, S. N., Erbs, G., Morrissey, K. L., Newman, M. A., Chinchilla, D., Boller, T., Molinaro, A., Jackson, R. W. and Cooper, R. M. (2009) Microbe-associated molecular pattern (MAMP) signatures, synergy, size and charge: influences on perception or mobility and host defence responses. *Mol. Plant Pathol.* **10**, 375-387
- 7 Bush, D. S. (1995) Calcium regulation in plant cells and its role in signaling. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 95-122
- 8 Mäser, P., Thomine, S., Schroeder, J. I., Ward, J. M., Hirschi, K., Sze, H., Talke, I. N., Amtmann, A., Maathuis, F. J., Sanders, D., Harper, J. F., Tchieu, J., Gribskov, M., Persans, M. W., Salt, D. E., Kim, S. A. and Guerinot, M. L. (2001) Phylogenetic relationships within cation transporter families of *Arabidopsis*. *Plant Physiol.* **126**, 1646-1667
- 9 Wheeler, G. L. and Brownlee, C. (2008) Ca<sup>2+</sup> signalling in plants and green algae - changing channels. *Trends Plant Sci.* **13**, 506-514
- 10 Chiu, J. C., Brenner, E. D., DeSalle, R., Nitabach, M. N., Holmes, T. C. and Coruzzi, G. M. (2002) Phylogenetic and expression analysis of the glutamate-receptor-like gene family in *Arabidopsis thaliana*. *Mol. Biol. Evol.* **19**, 1066-1082
- 11 Demidchik, V., Bowen, H. C., Maathuis, F. J., Shabala, S. N., Tester, M. A., White, P. J. and Davies, J. M. (2002) *Arabidopsis thaliana* root non-selective cation channels mediate calcium uptake and are involved in growth. *Plant J.* **32**, 799-808
- 12 Miedema, H., Demidchik, V., Very, A. A., Bothwell, J. H., Brownlee, C. and Davies, J. M. (2008) Two voltage-dependent calcium channels co-exist in the apical plasma membrane of *Arabidopsis thaliana* root hairs. *New Phytol.* **179**, 378-385
- 13 Ma, W., Qi, Z., Smigel, A., Walker, R. K., Verma, R. and Berkowitz, G. A. (2009) Ca<sup>2+</sup>, cAMP, and transduction of non-self perception during plant immune responses. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 20995-21000
- 14 Qi, Z., Verma, R., Gehring, C., Yamaguchi, Y., Zhao, Y., Ryan, C. A. and Berkowitz, G. A. (2010) Ca<sup>2+</sup> signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca<sup>2+</sup> channels. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 21193-21198
- 15 Clough, S. J., Fengler, K. A., Yu, I. C., Lippok, B., Smith, R. K., Jr. and Bent, A. F. (2000) The *Arabidopsis dnd1* "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 9323-9328
- 16 Jurkowski, G. I., Smith, R. K., Jr., Yu, I. C., Ham, J. H., Sharma, S. B., Klessig, D. F., Fengler, K. A. and Bent, A. F. (2004) *Arabidopsis DND2*, a second cyclic nucleotide-gated ion channel gene for which mutation causes the "defense, no death" phenotype. *Mol. Plant-Microbe Interact.* **17**, 511-520



- 17 Tikhonov, D. B. and Magazanik, L. G. (2009) Origin and molecular evolution of ionotropic glutamate receptors. *Neurosci. Behav. Physiol.* **39**, 763-773
- 18 Tapken, D. and Hollmann, M. (2008) *Arabidopsis thaliana* glutamate receptor ion channel function demonstrated by ion pore transplantation. *J. Mol. Biol.* **383**, 36-48
- 19 Chiu, J., DeSalle, R., Lam, H. M., Meisel, L. and Coruzzi, G. (1999) Molecular evolution of glutamate receptors: a primitive signaling mechanism that existed before plants and animals diverged. *Mol. Biol. Evol.* **16**, 826-838
- 20 Meyerhoff, O., Müller, K., Roelfsema, M. R., Latz, A., Lacombe, B., Hedrich, R., Dietrich, P. and Becker, D. (2005) *AtGLR3.4*, a glutamate receptor channel-like gene is sensitive to touch and cold. *Planta* **222**, 418-427
- 21 Michard, E., Lima, P. T., Borges, F., Silva, A. C., Portes, M. T., Carvalho, J. E., Gilliam, M., Liu, L. H., Obermeyer, G. and Feijo, J. A. (2011) Glutamate receptor-like genes form  $Ca^{2+}$  channels in pollen tubes and are regulated by pistil D-serine. *Science* **332**, 434-437
- 22 Dubos, C., Huggins, D., Grant, G. H., Knight, M. R. and Campbell, M. M. (2003) A role for glycine in the gating of plant NMDA-like receptors. *Plant J.* **35**, 800-810
- 23 Vatsa, P., Chiltz, A., Bourque, S., Wendehenne, D., Garcia-Brugger, A. and Pugin, A. (in press) Involvement of putative glutamate receptors in plant defence signaling and NO production. *Biochimie* (in press)
- 24 Knight, M. R., Campbell, A. K., Smith, S. M. and Trewavas, A. J. (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352**, 524-526
- 25 Rentel, M. C. and Knight, M. R. (2004) Oxidative stress-induced calcium signaling in *Arabidopsis*. *Plant Physiol.* **135**, 1471-1479
- 26 Saijo, Y., Tintor, N., Lu, X., Rauf, P., Pajeroska-Mukhtar, K., Haweker, H., Dong, X., Robatzek, S. and Schulze-Lefert, P. (2009) Receptor quality control in the endoplasmic reticulum for plant innate immunity. *EMBO J.* **28**, 3439-3449
- 27 Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K. and Scheible, W. R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* **139**, 5-17
- 28 Libault, M., Wan, J., Czechowski, T., Udvardi, M. and Stacey, G. (2007) Identification of 118 *Arabidopsis* transcription factor and 30 ubiquitin-ligase genes responding to chitin, a plant-defense elicitor. *Mol. Plant-Microbe Interact.* **20**, 900-911
- 29 Boudsocq, M., Willmann, M. R., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, S. H. and Sheen, J. (2010) Differential innate immune signalling via  $Ca^{2+}$  sensor protein kinases. *Nature* **464**, 418-422
- 30 Felix, G., Regenass, M. and Boller, T. (1993) Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein-phosphorylation, and establishment of a refractory state. *Plant J.* **4**, 307-316
- 31 Lecourieux, D., Lamotte, O., Bourque, S., Wendehenne, D., Mazars, C., Ranjeva, R. and Pugin, A. (2005) Proteinaceous and oligosaccharidic elicitors induce different calcium signatures in the nucleus of tobacco cells. *Cell Calcium* **38**, 527-538
- 32 de Jong, C. F., Laxalt, A. M., Bargmann, B. O., de Wit, P. J., Joosten, M. H. and Munnik, T. (2004) Phosphatidic acid accumulation is an early response in the *Cf-4/Avr4* interaction. *Plant J.* **39**, 1-12
- 33 Munnik, T. and Testerink, C. (2009) Plant phospholipid signaling: "in a nutshell". *J. Lipid Res.* **50 Suppl**, S260-265
- 34 Endo, M. (2009) Calcium-induced calcium release in skeletal muscle. *Physiol. Rev.* **89**, 1153-1176

- 35 Pineros, M. and Tester, M. (1997) Calcium channels in higher plant cells: selectivity, regulation and pharmacology. *J. Exp. Bot.* **48**, 551-577
- 36 Pottosin, II, Dobrovinskaya, O. R. and Muniz, J. (1999) Cooperative block of the plant endomembrane ion channel by ruthenium red. *Biophys. J.* **77**, 1973-1979
- 37 Foster, A. C. and Wong, E. H. (1987) The novel anticonvulsant MK-801 binds to the activated state of the N-methyl-D-aspartate receptor in rat brain. *Brit. J. Pharmacol.* **91**, 403-409
- 38 Bertolino, M., Vicini, S. and Costa, E. (1989) Kynurenic acid inhibits the activation of kainic and N-methyl-D-aspartic acid-sensitive ionotropic receptors by a different mechanism. *Neuropharmacology* **28**, 453-457
- 39 Honore, T., Davies, S. N., Drejer, J., Fletcher, E. J., Jacobsen, P., Lodge, D. and Nielsen, F. E. (1988) Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science* **241**, 701-703
- 40 Stephens, N. R., Qi, Z. and Spalding, E. P. (2008) Glutamate receptor subtypes evidenced by differences in desensitization and dependence on the *GLR3.3* and *GLR3.4* genes. *Plant Physiol.* **146**, 529-538
- 41 Demidchik, V., Essah, P. A. and Tester, M. (2004) Glutamate activates cation currents in the plasma membrane of *Arabidopsis* root cells. *Planta* **219**, 167-175
- 42 Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M. and Hermann Muehling, K. (2001) Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiol. Plant.* **111**, 457-465
- 43 Forde, B. G. and Lea, P. J. (2007) Glutamate in plants: metabolism, regulation, and signalling. *J. Exp. Bot.* **58**, 2339-2358
- 44 Pitzschke, A., Schikora, A. and Hirt, H. (2009) MAPK cascade signalling networks in plant defence. *Curr. Opin. Plant Biol.* **12**, 421-426
- 45 Jeworutzki, E., Roelfsema, M. R., Anschütz, U., Krol, E., Elzenga, J. T., Felix, G., Boller, T., Hedrich, R. and Becker, D. (2010) Early signaling through the *Arabidopsis* pattern recognition receptors FLS2 and EFR involves  $\text{Ca}^{2+}$ -associated opening of plasma membrane anion channels. *Plant J.* **62**, 367-378
- 46 Nagai, T., Yamada, S., Tominaga, T., Ichikawa, M. and Miyawaki, A. (2004) Expanded dynamic range of fluorescent indicators for  $\text{Ca}^{2+}$  by circularly permuted yellow fluorescent proteins. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 10554-10559
- 47 Monshausen, G. B., Messerli, M. A. and Gilroy, S. (2008) Imaging of the yellow cameleon 3.6 indicator reveals that elevations in cytosolic  $\text{Ca}^{2+}$  follow oscillating increases in growth in root hairs of *Arabidopsis*. *Plant Physiol.* **147**, 1690-1698
- 48 Rincón-Zachary, M., Teaster, N. D., Sparks, J. A., Valster, A. H., Motes, C. M. and Blancaflor, E. B. (2010) Fluorescence resonance energy transfer-sensitized emission of yellow cameleon 3.60 reveals root zone-specific calcium signatures in *Arabidopsis* in response to aluminum and other trivalent cations. *Plant Physiol.* **152**, 1442-1458
- 49 Qi, Z., Stephens, N. R. and Spalding, E. P. (2006) Calcium entry mediated by *GLR3.3*, an *Arabidopsis* glutamate receptor with a broad agonist profile. *Plant Physiol.* **142**, 963-971
- 50 Wurzinger, B., Mair, A., Pfister, B. and Teige, M. (2011) Cross-talk of calcium-dependent protein kinase and MAP kinase signaling. *Plant Signal. Behav.* **6**, 8-12

## Figure legends

### Figure 1 flg22, elf18 and chitin induce $\text{Ca}^{2+}$ influx from the apoplast.

10 to 12-day-old aequorin-expressing *Arabidopsis* seedlings were pretreated for one hour with 1 mM  $\text{LaCl}_3$  (A-C) or 10 mM EGTA (D). The measurements were started and after two minutes, flg22 (1  $\mu\text{M}$ , A and D), elf18 (1  $\mu\text{M}$ , B) or chitin (0,1 mg/ml, C) were automatically added. The luminescence originating from the seedlings was followed in time and  $\text{Ca}^{2+}$  concentrations were calculated according to [25]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are average  $\Delta[\text{Ca}^{2+}]$  values ( $\pm$  standard deviation) after correction for basal  $\text{Ca}^{2+}$  levels before MAMP addition. Curves shown are based on representative measurements of 4 to 6 seedlings per treatment. Each experiment was at least performed three times with similar results.

### Figure 2 No apparent contribution from internal $\text{Ca}^{2+}$ stores and VGCCs to the flg22-, elf18- and chitin-induced $\Delta[\text{Ca}^{2+}]$ .

10 to 12-day-old aequorin-expressing *Arabidopsis* seedlings were pretreated for one hour with 10  $\mu\text{M}$  U73122 (A and B), 200  $\mu\text{M}$  Ruthenium Red (C and D), 100  $\mu\text{M}$  tetracaine (E) or 100  $\mu\text{M}$  nifedipine (F). The measurements were started and after two minutes, flg22 (1  $\mu\text{M}$ , A, B, E and F), elf18 (1  $\mu\text{M}$ , C) or chitin (0,1 mg/ml, D) were automatically added. The luminescence originating from the seedlings was followed in time and  $\text{Ca}^{2+}$  concentrations were calculated according to [25]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are average  $\Delta[\text{Ca}^{2+}]$  values ( $\pm$  standard deviation) after correction for basal  $\text{Ca}^{2+}$  levels before MAMP addition (B-F) or absolute  $[\text{Ca}^{2+}]$  (A). Curves shown are based on representative measurements of 4 to 6 seedlings per treatment. Each experiment was at least performed three times with similar results.

### Figure 3 Modulation of cNTP signalling does not influence the flg22-, elf18- or chitin-induced $\Delta[\text{Ca}^{2+}]$ .

10 to 12-day-old aequorin-expressing *Arabidopsis* seedlings were pretreated for one hour with 1 mM alloxan (A), 200  $\mu\text{M}$  dideoxyadenosine (B). The measurements were started and after two minutes, flg22 (1  $\mu\text{M}$ ) was automatically added. The luminescence originating from the seedlings was followed in time and  $\text{Ca}^{2+}$  concentrations were calculated according to [25]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are average  $\Delta[\text{Ca}^{2+}]$  values ( $\pm$  standard deviation) after correction for basal  $\text{Ca}^{2+}$  levels before MAMP addition. Curves shown are based on representative measurements of 4 to 6 seedlings per treatment. Each experiment was at least performed three times with similar results.

### Figure 4 Modulation of iGluR-like channel function reduces the flg22-, elf18- or chitin-induced $\Delta[\text{Ca}^{2+}]$ .

10 to 12-day-old aequorin-expressing *Arabidopsis* seedlings were pretreated for one hour with 1 mM AP-5 (A and B), 1 mM kynurenic acid (C), 1 mM L-glutamate (D and E), different amino acids (F) or a range of L-glutamate concentrations (G). The measurements were started and after two minutes, flg22 (1  $\mu\text{M}$ ) was automatically added. The luminescence originating from the seedlings was followed in time and  $\text{Ca}^{2+}$  concentrations were calculated according to [25]. Shown are average  $\Delta[\text{Ca}^{2+}]$  values ( $\pm$  standard deviation) after correction for basal  $\text{Ca}^{2+}$  levels before MAMP addition (B, C and E) or absolute  $[\text{Ca}^{2+}]$  (A, D). The histogram (F) shows the steady state  $[\text{Ca}^{2+}]$  or the  $\Delta[\text{Ca}^{2+}]$  (nM) after flg22 (1  $\mu\text{M}$ ), elf18 (1  $\mu\text{M}$ ) or chitin (0.1 mg/ml) stimulus at the maximum of the  $\text{Ca}^{2+}$  transient after pretreatment for 1 hour with 1mM L-alanine [A], L-cysteine [C], L-aspartate [D], L-glutamate [E], glycine [G], L-histidine [H], L-lysine [K], L-leucine [L], L-glutamine [Q], L-tryptophan [W] and the mock control [-]. Values represent averages of 3 to 6 biological replicates with 6 individual seedlings per replicate. Statistical significant differences were assessed using a Student's t-test with treatments compared to the mock control ( $P < 0.05$ ) (G). For the L-glutamate concentration range (G), the average basal  $\text{Ca}^{2+}$  concentration was determined using the average  $\text{Ca}^{2+}$  concentration of the time points before injecting flg22. The  $\Delta\text{Ca}^{2+}$  value was calculated using background-corrected  $\text{Ca}^{2+}$  concentrations and the values between 3.5 and 5 minutes were averaged. Each experiment was at least performed three times with similar results. Curves shown are based on representative measurements of

iGluRs mediate MAMP-triggered calcium influx in *Arabidopsis*

4 to 6 seedlings per treatment (A-E) or the mean of  $n=3$  independent experiments (F). Error bars in the graphs represent the SD (A-E) or the standard error of the mean (F-G).

**Figure 5 Modulation of iGluR-like channel function or inhibition of  $\text{Ca}^{2+}$  influx from the apoplast affects MAPK activation.**

10-12-day-old *Arabidopsis* Col-0 seedlings were pretreated for one hour with 1 mM L-glutamate (A-C), 1 mM kynurenic acid (A and B), 1 mM CNQX (A and B), 1 mM  $\text{LaCl}_3$  (C) or 10 mM EGTA (C). Time point 0 minutes was harvested before MAMP addition. 1 mM L-glutamate (A) or 0,1 mg/ml chitin (B and C) were added and after 5 minutes and 15 minutes samples were taken and analysed for MAPK activation as previously described [26]. Per sample 10-12 seedlings were used. Arrows mark the activated MAPKs on the blots. Experiments were repeated two or three times with similar results.

**Figure 6 iGluR signalling and cNTP signalling act additively in elf18-induced  $\text{Ca}^{2+}$  influx.**

10-12-day-old aequorin expressing *Arabidopsis* seedlings (A) and Col-0 wild type seedlings (B) were pretreated for one hour with 1 mM L-glutamate, 1 mM alloxan or a combination of both. Aequorin based  $\text{Ca}^{2+}$  measurements (A): Two minutes after initiation of luminescence detection, elf18 (1  $\mu\text{M}$ ) was automatically added. The luminescence originating from the seedlings was followed in time and  $\text{Ca}^{2+}$  concentrations were calculated according to [25]. The grey marking, delineates the spike in luminescence caused by the injection of the MAMPs. Shown are the averages of the mean  $\Delta[\text{Ca}^{2+}]$  of  $>3$  biological replicates per treatment after correction for base  $\text{Ca}^{2+}$  levels with the standard error of mean. MAPK assays (B): Time point 0 minutes was harvested before MAMP addition; then, 1  $\mu\text{M}$  elf18 was added and after 5 minutes and 15 minutes samples were taken and analysed for MAPK activation as previously described [26]. Per sample 10-12 seedlings were used. Arrows mark the activated MAPKs on the blots. Experiments were repeated two or three times with similar results.

**Figure 7 Modulation of iGluR-mediated  $\text{Ca}^{2+}$  influx inhibits MAPK- and CDPK-dependent defense gene transcript accumulation.**

10-12-day-old *Arabidopsis* Col-0 wild type seedlings were pretreated for one hour with 1 mM L-glutamate or 1 mM kynurenic acid. Time point 0 minutes was harvested before MAMP addition and flg22 (1  $\mu\text{M}$ ), elf18 (1  $\mu\text{M}$ ) or chitin (0,1 mg/ml) were added. Samples were taken at 30 and 60 minutes after MAMP addition and prepared for quantitative RT-PCR. PCRs were performed with primers specific for FRK1 and PHI-1 [29] and the reference gene At4G26410. The data were normalized to the reference gene and the value at time point 0 minutes of the non-pretreated samples was set to 1. The mean relative transcript accumulation of FRK1 (A) and PHI-1 (B) are presented in the graphs. Error bars represent the standard deviation of three technical replicates. The experiment was repeated three times yielding similar results.

**Figure 8 A model for MAMP-triggered iGluR-mediated  $\text{Ca}^{2+}$  influx in *Arabidopsis thaliana*.**

The scheme depicts a potential scenario based on our experimental results.  $\text{CaM-Ca}^{2+}$ , activated ( $\text{Ca}^{2+}$ -bound) calmodulin. For further explanation see main text.

**Table 1 Overview of alterations in intracellular Ca<sup>2+</sup> levels upon MAMP treatment in the presence of compounds modulating Ca<sup>2+</sup> transport <sup>a</sup>.**

Pre-treatment	flg22			elf18			Chitin			Action
	Base	Peak	Rec	Base	Peak	Rec	Base	Peak	Rec	
-	=	=	=	=	=	=	=	=	=	
LaCl <sub>3</sub>	=	0	=	=	0	=	=	0	=	Blocks Ca <sup>2+</sup> influx channels
EGTA	↑	0	=	↑	0	=	↑	0	=	Chelates extracellular Ca <sup>2+</sup>
U73122	↑	=	=	↑	=	=	↑	=	=	Inhibits phospholipase C
Neomycin	↑	↓	=	↑	↓	=	↑	=	=	Inhibits phospholipase C
Ruthenium Red	↑	↓	=	↑	↓	=	↑	↓↓	=	Inhibits ryanodine receptor-controlled CICR and influx via plasma membrane-resident channels
Tetracaine	=	↑	= ^^	=	↑	= ^^	=	↑	= ^^	Inhibits ryanodine receptor-controlled CICR release
Diltiazem	=	=	= ^^	=	=	= ^^	=	=	= ^^	L-Type VGCC inhibitor
Verapamil	=	=	= ^^	=	=	= ^^	=	=	= ^^	L-Type VGCC inhibitor
Nifedipine	=	=	→	=	↓	→	=	↓	→	L-Type VGCC inhibitor plus inhibitor of ABC transporters
Alloxan	=	=	=	=	↓	=	=	↓	=	Adenylate cyclase inhibitor
Dideoxyadenosine	=	=	=	=	↓	=	=	↓	=	Adenylate cyclase inhibitor
L-glutamate	↑	↓↓	=	↑	↓↓	=	↑	↓↓	=	iGluR agonist
AP-5	↑	↓↓	=	↑	↓↓	=	↑	↓↓	=	iGluR NMDA antagonist (L-glutamate binding site)
AP-7	↑	↓↓	=	NT	NT	NT	NT	NT	NT	iGluR NMDA antagonist (L-glutamate binding site)
Kynurenic acid (KA)	↑	↓↓	=	↑	↓↓	=	↑	↓↓	=	iGluR non-selective NMDA/non-NMDA antagonist (glycine binding site)
MK-801	=	=	=	=	=	=	=	=	=	iGluR NMDA receptor antagonist (binds in pore)
DNQX	=	=	=	=	=	=	=	=	=	iGluR non-NMDA receptor antagonist (binds in pore)
CNQX	=	=	=	=	=	=	=	↓	=	iGluR non-NMDA receptor antagonist (binds in pore)
Alloxan + L-glutamate	↑	↓↓	=	↑	↓↓	=	↑	↓↓	=	
Alloxan + KA	↑	↓↓	=	↑	↓↓	=	↑	↓↓	=	
DDA + L- glutamate	↑	↓↓	=	↑	↓↓	=	↑	↓↓	=	
DDA + KA	↑	↓↓	=	↑	↓↓	=	↑	↓↓	=	

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111112



<sup>a</sup>Qualitative evaluation of response of at least 3 replicates. = same as non-treated, 0 no Ca<sup>2+</sup> transient visible, ↑ [Ca<sup>2+</sup>] increased compared to non-treated, → delayed recovery [Ca<sup>2+</sup>] after application MAMP compared to non-treated, ↓ small reduction Δ[Ca<sup>2+</sup>] in peak compared to non-treated, ↓↓ strong reduction Δ[Ca<sup>2+</sup>] in peak compared to non-treated, ^^ occurrence of random increases in [Ca<sup>2+</sup>] during recovery phase after MAMP application, NT, not tested

Accepted Manuscript

Figure 1

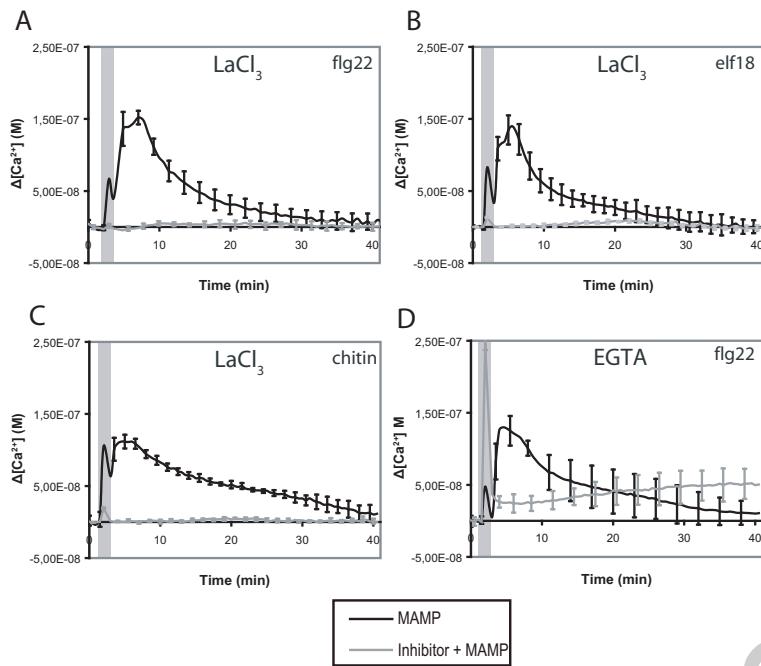


Figure 2

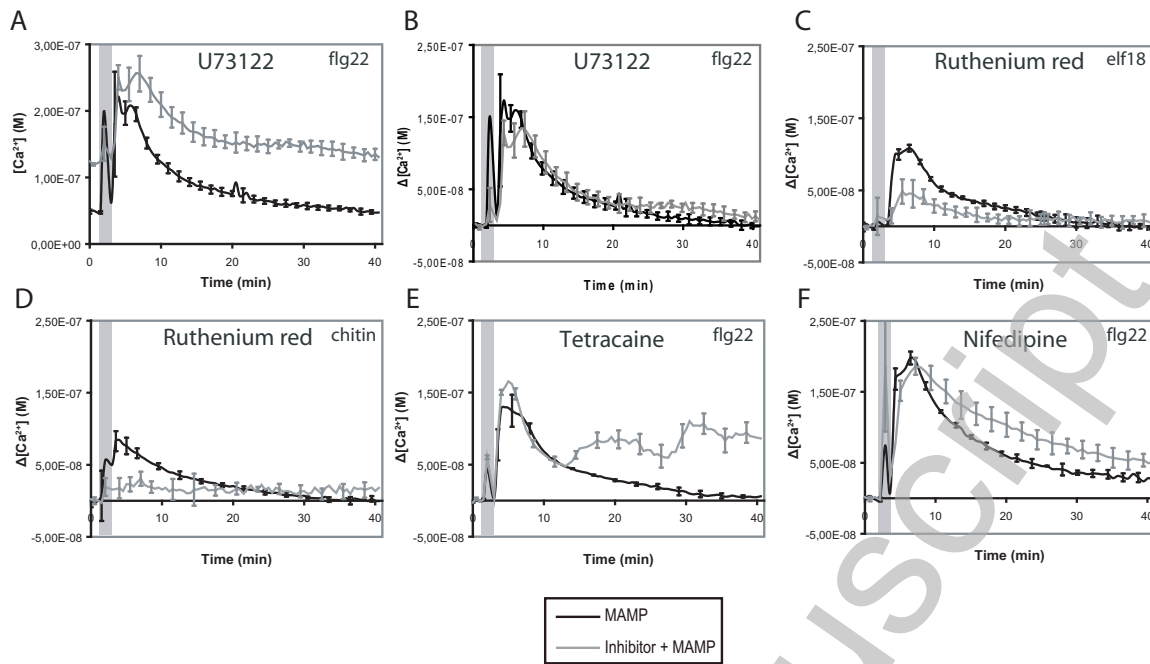
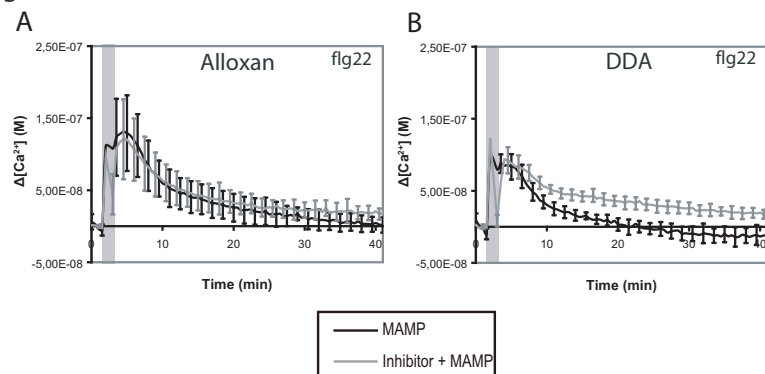


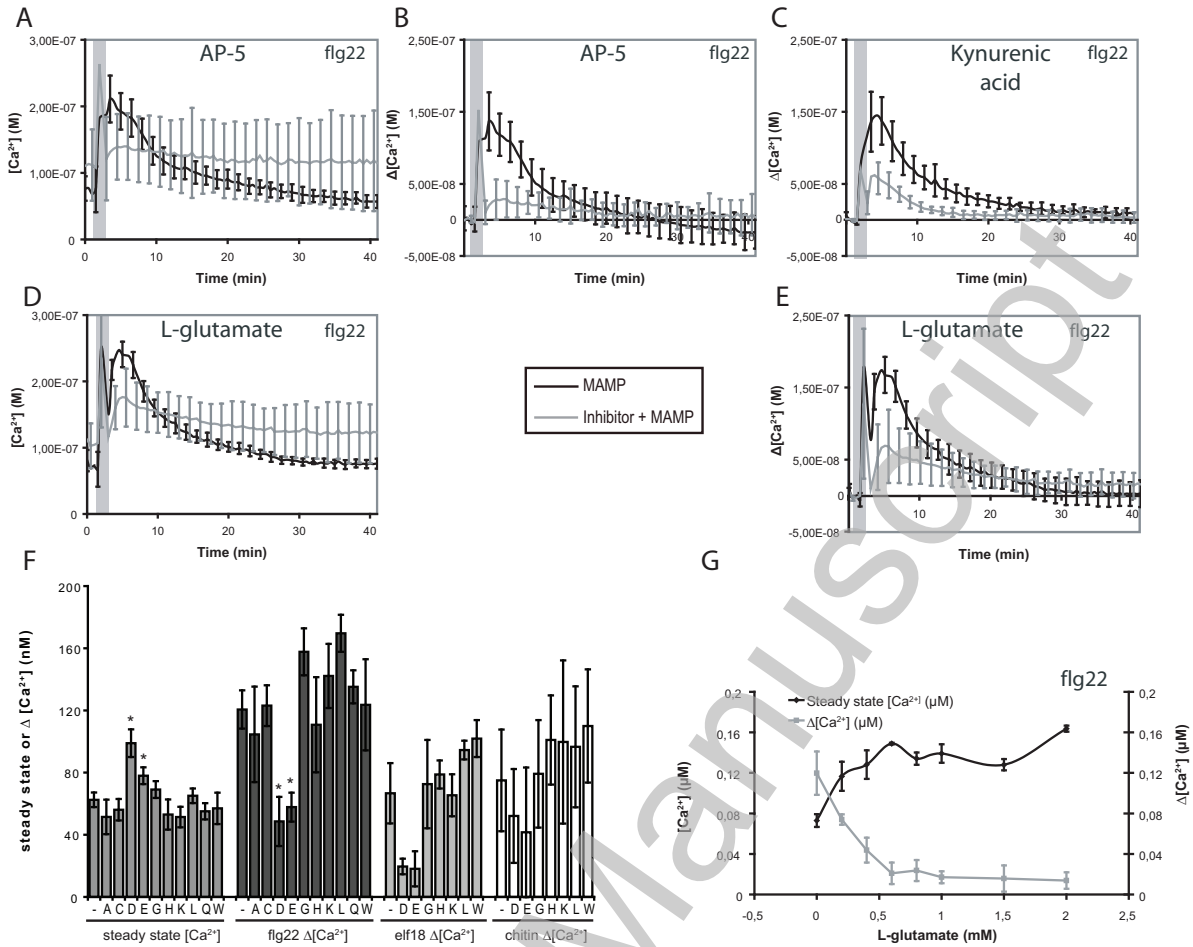
Figure 3



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111112

Accepted Manuscript

Figure 4

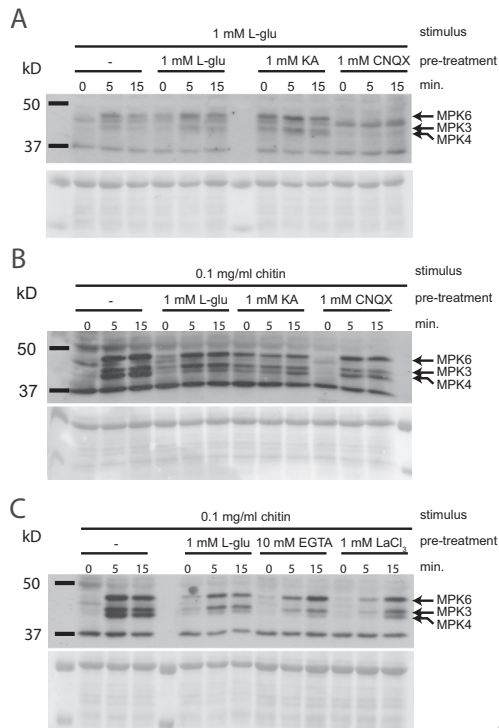


THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111112

Accepted Manuscript



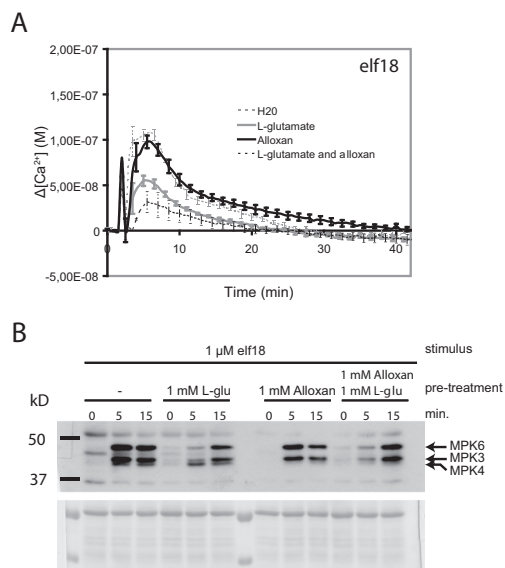
Figure 5



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111112

Accepted Manuscript

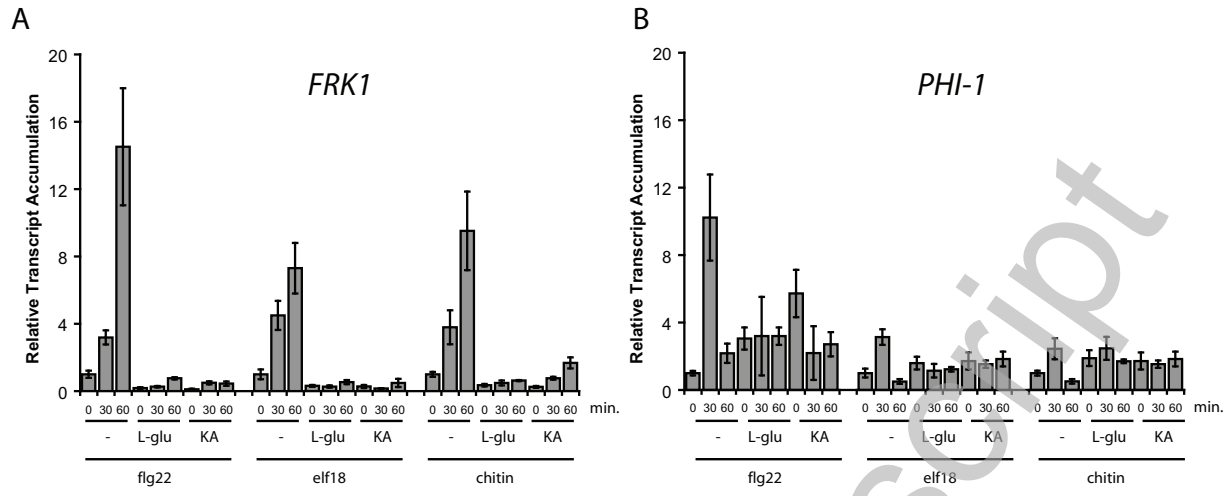
Figure 6



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111112

Accepted Manuscript

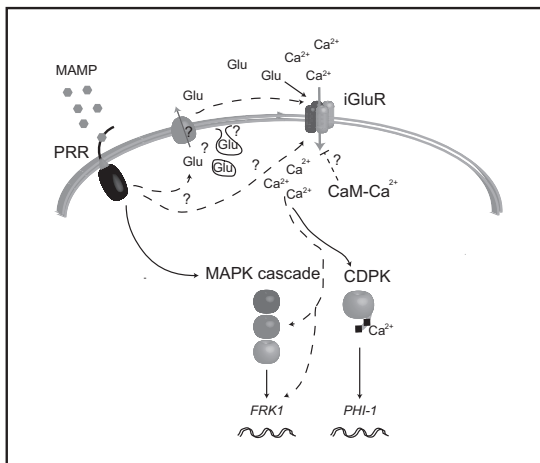
Figure 7



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111112

Accepted Manuscript

Figure 8



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111112

Accepted Manuscript