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

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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 PRRs (pattern recognition receptors) that sense specific microbial epitopes termed MAMPs (microbe-associated molecular patterns). PRRs play a key role in plant innate immunity through the activation of defence response pathways upon MAMP sensing (MAMP-triggered immunity) [1]. Examples of PRRs comprise FLS2 (flagellin sensing 2), EFR [EF-Tu (elongation factor-thermo unstable) receptor] and CERK1 (chitin elicitor receptor kinase 1). FLS2 and EFR activate downstream signalling 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 Ca2+ and other ions, the generation of ROS (reactive oxygen species), the initiation of MAPK (mitogen-activated protein kinase) cascades and the activation of CDPKs (Ca2+-dependent protein kinases) [1]. Despite their canonical occurrence, the functional role of these archetypal responses in plant innate immunity remains largely obscure.

Ca2+-transport processes in plants and animals we found strong evidence for a prominent role of amino acid-controlled Ca2+ fluxes, probably through iGluR (ionotropic glutamate receptor)-like channels. Interference with amino acid-mediated Ca2+ fluxes modulates MAMP-triggered MAPK (mitogen-activated protein kinase) activity and affects MAMP-induced accumulation of defence gene transcripts. We conclude that the initiation of innate immune responses upon flg22, elf18 and chitin recognition involves apoplastic Ca2+ influx via iGluR-like channels.

Key words: calcium channel, calcium signature, elicitor, ionotropic glutamate receptor channel, microbe-associated molecular pattern, plant immunity.

Binding of specific microbial epitopes [MAMPs (microbe-associated molecular patterns)] to PRRs (pattern recognition receptors) 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 Ca2+ levels. In plants, knowledge about the signalling events leading to Ca2+ influx and on the molecular identity of the channels involved is scarce. We used a transgenic Arabidopsis thaliana line stably expressing the luminescent aequorin Ca2+ biosensor to monitor pharmacological interference with Ca2+ 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 Ca2+-transport processes in plants and animals we found strong evidence for a prominent role of amino acid-controlled Ca2+ fluxes, probably through iGluR (ionotropic glutamate receptor)-like channels. Interference with amino acid-mediated Ca2+ fluxes modulates MAMP-triggered MAPK (mitogen-activated protein kinase) activity and affects MAMP-induced accumulation of defence gene transcripts. We conclude that the initiation of innate immune responses upon flg22, elf18 and chitin recognition involves apoplastic Ca2+ influx via iGluR-like channels.

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INTRODUCTION

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Ca2+ 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 Ca2+ signatures; transients that differ in timing as well as the number and magnitude of Ca2+ spikes. The transient increase of cytosolic [Ca2+] results from passive Ca2+ influx from the apoplast or intracellular Ca2+ stores through dedicated channels [7]. Since Ca2+ is cytotoxic, low concentrations are maintained in the cytosol and nucleus of resting cells. H+/Ca2+-exchangers and Ca2+-ATPases (ATP-dependent Ca2+ pumps) expel Ca2+ in an energy-dependent process from the cytosol to the extracellular space and into intracellular compartments such as the vacuole and the ER (endoplasmic reticulum). As a result, the [Ca2+] 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 Ca2+ into the cytosol [7].

Genome-wide comparisons revealed large differences between families for channel candidates mediating Ca2+ import in plants and mammals [8–10]. Genes encoding obvious orthologues of some major classes of mammalian channels are lacking in plants (ryanodine receptor channels, InsP3-activated receptor channels and voltage-gated channels), whereas the family of CNGCs (cyclic nucleotide-gated channels) is expanded in plants. Even though sequence-related genes are missing, electrophysiological experiments indicated the presence of voltage-gated Ca2+ channels in plant organelles and cells [11,12]. Apparently, a unique and sequence-diverged set of proteins contributes to the control of Ca2+ influx into plant cells.

Abbreviations used: CaM, calmodulin; CDPK, Ca2+-dependent protein kinase; CERK1, chitin elicitor receptor kinase 1; CICR, Ca2+-induced Ca2+ release; CNGC, cyclic nucleotide-gated channel; cNTP, cyclic nucleotide; CNQX, 6-cyano-7-nitroquinazoline-2,3-dione; DDA, dideoxynudenosine; ddH2O, double-distilled water; dnf, defence no death; DNQX, 6,7-dinitroquinazoline-2,3-dione; FLS2, flagellin sensing 2; FRK1 flg22-induced-receptor-like-kinase 1; HR, hypersensitive response; iGluR, ionotropic glutamate receptor; LPS, lipopolysaccharide; MAMP, microbe-associated molecular pattern; MAPK, mitogen-activated protein kinase; MAPK(K/KK), MAPK cascade; NMDA, N-methyl-D-aspartate; PHI-1, phosphate-induced 1; PLC, phospholipase C; PRR, pattern recognition receptor; RR, Ruthenium Red; U73122, 1-{[17-3-methoxyestra-1,3,5(10)-trien-17-y]amino}(hexyl)-1H-pyrrrole-2,5-dione; VGCC, voltage-gated Ca2+ channel.

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CNGCs comprise a family of 20 ligand-gated ion channels in Arabidopsis thaliana [8]. CNGCs are gated by cNTPs (cyclic nucleotides) [13,14]. Several CNGCs have been functionally linked to plant immunity. For example, the dnd (defence no death) class of mutants has a reduced ability to initiate a HR (hypersensitive response) to avirulent bacteria. DND1 and DND2 encode two CNGC paralogues (CNGC2 and CNGC4) [15,16]. In addition to the altered cell-death phenotype, cngc2-knockout plants show an altered Ca\(^{2+}\) signature in response to the bacterial MAMP LPS (lipopolysaccharide) [13].

The second major class of putative ligand-gated Ca\(^{2+}\)-permeable channels in plants comprises the iGluR (ionotropic glutamate receptor)-like channels. 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 iGluR-like channels (AtiGluR1.1 and AtiGluR1.4) are permeable to Ca\(^{2+}\), Na\(^+\) and K\(^+\) [18]. The Arabidopsis genome encodes 20 iGluR-like channels [9,10,19]. They are involved in various processes ranging from biotic- and abiotic-stress responses [20] to pollen-tube growth [21] and hypocotyl elongation [22]. Recently, Ca\(^{2+}\) influx and NO production triggered by the proteinaceous oomycete MAMP cryptogein were also found to involve iGluR-like channels [23].

In the present study we used a transgenic A. thaliana cell line expressing the aequorin Ca\(^{2+}\) biosensor to investigate intracellular Ca\(^{2+}\) signatures in response to three different well-characterized MAMPs (flg22, elf18 and chitin). We conducted pharmacological interference with the MAMP-triggered Ca\(^{2+}\) influx using a comprehensive set of compounds known to impede various classes of Ca\(^{2+}\) channels. Besides the actual Ca\(^{2+}\) signatures, we used MAMP-induced downstream responses such as MAPK activation and accumulation of defence gene transcripts as additional readouts for Ca\(^{2+}\)-channel modulation. The findings of the present study support a crucial role for iGluR-like channels in the generation of MAMP-triggered Ca\(^{2+}\) signatures and provide evidence for a critical role for MAMP-induced Ca\(^{2+}\) signalling in defence 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 on to a 96-well plate (PerkinElmer; 1–2 seeds per well) containing 100 \(\mu\)l of 1xMS (Murashige and Skoog) medium including vitamins (Duchefa) with 0.25% sucrose. The seedlings were grown for 11–12 days with 16 h light (at 21°C), 8 h dark (at 19°C) before further study.

Chemicals

Unless stated otherwise, chemicals were obtained from Sigma or Tocris Biosciences. Stock solutions were prepared in ddH\(_2\)O (double-distilled water) except for CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), U73122 [1-{6-[[17,3-methoxyestra-1,3,5(10)-trien-17-y]aminol]-1H-pyrrole-2,5-dione] 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.

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 \(\mu\)l ddH\(_2\)O containing 10 \(\mu\)M coelenterazine (Biosynth), diluted from a 5 mM stock solution in methanol, and incubated overnight in the dark. The next day, the liquid was replaced by 100 \(\mu\)l of ddH\(_2\)O and the seedlings were incubated for 30 min in the dark. For the compound treatments, 10-fold concentrated stocks in water were prepared and added to the coelenterazine-loaded seedlings. The seedlings were pre-incubated for 1 h before starting the measurements. Elf18 and flg22 were present as 10 or 1 mM stock solutions in water stored at \(-80^\circ\)C and freshly used. Chitin solution was prepared by intermittently grinding (for 15–20 min) 10 mg of crab or shrimp shell powder (Sigma) in 1 ml of ddH\(_2\)O with a pestle and subsequent vortexing and heating the solution to 60°C. Undissolved particles were removed by centrifugation (15 min, 15,700 \(\times\)g at 25°C) and the clear supernatant was used for the experiments. The measurements were performed in a 96-well Centro LB960 luminometer system (Berthold Technologies). Half a plate was measured in one go. Luminescence from one single well was detected for 0.25 s and each well was measured every 30 s. After 2 min (four measurements), 5-fold concentrated MAMP was automatically added to a final concentration of 1 \(\mu\)M flg22, 1 \(\mu\)M elf18 or 0.1 mg/ml chitin. After this addition, the luminescence was followed for an additional 40 min. The second half of the plate was run in the same manner. Finally, for calculation of Ca\(^{2+}\) concentrations, 100 \(\mu\)l of 2 M CaCl\(_2\) in 20% ethanol was added to each well using the injectors of the system and the luminescence was measured for 0.25 s per well with one measurement per well every 63 s. The luminescence was followed for 30 min. Ca\(^{2+}\) concentrations were calculated using the method of Rentel and Knight [25].

Per treatment, four to six individual wells were averaged. Ca\(^{2+}\) transients were compared between treatments within one experiment unless stated otherwise and at least three biological replicates per compound were performed. Unless otherwise stated, the results were normalized to background Ca\(^{2+}\) levels and represented as a \(\Delta [Ca^{2+}]\) value to compensate for background variations between measurements. Owing to the low molecular mass of aequorin (22 kDa) and its presumed passive transfer into the nucleus, we expect the Arabidopsis reporter line to monitor the combined cytoplasmic and nuclear (in the present paper referred to as intracellular) Ca\(^{2+}\) pool. The statistical significance of differences was evaluated by comparing average Ca\(^{2+}\) 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 described previously [26].

Quantitative RT (reverse transcription)-PCR

Seedlings were grown and pretreated as described for the Ca\(^{2+}\) measurements, excluding the overnight incubation step with coelenterazine. Per treatment, 10–15 seedlings were used and the MAMP stimulus was added manually. Time point 0 was harvested before MAMP addition and 30 and 60 min after MAMP addition.
Total RNA was isolated using the RNeasy Plant Kit (Qiagen). cDNA was synthesized with 5 μg of total RNA, oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen) according to manufacturer’s protocol. Transcript abundance was quantified using the iQ5 real-time PCR thermocycler (Bio-Rad Laboratories). The expression of the genes of interest was normalized to A14260410 (for the primers used see [27]) and to the relative transcript abundance in non-treated Col-0 control samples as calculated by the comparative-cycle threshold (ΔΔCt) method [28]. Transcript abundance of a MAPK-dependent gene FRK1 (flg22-induced-receptor-like-kinase 1) and a CDPK-dependent gene PHI-1 (phosphate-induced 1) were tested (for the primers used see [29]). Three biological replicates with three technical replicates per sample were performed per treatment.

RESULTS

MAMP-triggered Ca2+ influx from the apoplast

We used the luminescent aequorin Ca2+-sensor system [24] to record whole-seedling intracellular Ca2+ transients in response to MAMPs. On the basis of 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 Ca2+ signatures in Arabidopsis, a range of chemicals were chosen that are known to target components controlling Ca2+ influx and efflux in plant and animal systems. Owing to potential plant etal, 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 [Ca2+] rise, the general Ca2+ channel inhibitor LaCl3 (lanthanum chloride) and a chelator of extracellular bivalent cations, EGTA, were applied. Consistent with previous reports [6,31], flg22 and elf18 induced a biphasic Ca2+ response with a maximum [Ca2+] at approximately 3 min after MAMP addition and a recovery phase of approximately 20–30 min (Figures 1A and 1B). Chitin induced a monophasic Ca2+ transient (Figure 1C). Pre-incubation of the seedlings with either LaCl3 or EGTA fully inhibited the Ca2+ transients induced by all three MAMPs, suggesting that the main Ca2+ source for the MAMP-triggered spikes is the apoplast (Figures 1A–1D and Table 1, and Supplementary Figures S1A and S1B at http://www.BiochemJ.org/bj/440/bj4400355add.htm).

In the presence of EGTA, a continuous rise in intracellular Ca2+ levels was observed, independent of the presence or absence of MAMPs (Figure 1D, and Supplementary Figures S1A and S1B).

An ambiguous role for internal Ca2+ stores in MAMP-triggered Ca2+ influx

To further validate the conclusion that the major source of MAMP-triggered Ca2+ is the apoplast, we studied the role of PLC (phospholipase C)-dependent release of Ca2+ from intracellular stores using the PLC inhibitors neomycin and U73122 [32,33]. Both inhibitors led to increased steady-state Ca2+ levels (Figure 2A and Table 1, and Supplementary Figure S2C at http://www.BiochemJ.org/bj/440/bj4400355add.htm). U73122 did not alter Δ[Ca2+] for the MAMPs tested, but changed the shape of the response curves of elf18 and chitin (Figure 2B, and Supplementary Figures S2A and S2B), which suggests a minor contribution for PLC-controlled Ca2+ release in response to these MAMPs. Neomycin did not alter the chitin-induced Δ[Ca2+] (Supplementary Figure S2F), but caused a slight reduction in the flg22- and elf18-induced Δ[Ca2+] (Supplementary Figures S2C–S2E). In conclusion, PLC-mediated Ca2+ release has a minor, if any, contribution to the MAMP-triggered Δ[Ca2+].

A MAMP-triggered primary apoplastic Ca2+ influx does not exclude the possibility of secondary CICR (Ca2+-induced Ca2+ release) from internal stores. RR (Ruthenium Red) 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 [Ca2+] (Table 1 and Supplementary Figure S2G). In conclusion, PLC-mediated Ca2+ release has a minor, if any, contribution to the MAMP-triggered Δ[Ca2+].

Figure 1  Flg22, elf18 and chitin induce Ca2+ influx from the apoplast

Aquaporin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 1 mM LaCl3 (A–C) or 10 mM EGTA (D). The measurements were started and after 2 min flg22 (1 μM; A and D), elf18 (1 μM; B) or chitin (0.1 mg/ml; C) were automatically added. The luminescence originating from the seedlings was followed in time and Ca2+ concentrations were calculated as described previously [25]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean ± S.D. Δ[Ca2+] values after correction for basal Ca2+ levels before MAMP addition. Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.
Inhibitors of voltage- and nucleotide-gated channels do not affect MAMP-triggered Ca\(^{2+}\) influx

To further delimit the channels involved in Ca\(^{2+}\) influx from extracellular stores, a set of inhibitors targeting animal type VGCC (voltage-gated Ca\(^{2+}\) channels) 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 Δ[Ca\(^{2+}\)] triggered by the MAMP stimulus (Figure 2F and Table 1, and Supplementary Figures S3A–S3H at http://www.BiochemJ.org/bj/440/bj4400355add.htm). Nifedipine treatment resulted in a delayed recovery in the case of all three MAMPs (Figure 2F and Table 1, and Supplementary Figures S3A–S3B). Verapamil-treated seedlings occasionally showed random spiking after the MAMP stimulus (Table 1 and Supplementary Figures S3C–S3E). Taken together, these results showed random spiking after the MAMP stimulus (Table 1 and Figures S3A–S3B). V erapamil-treated seedlings occasionally showed random spiking after the MAMP stimulus (Table 1 and Figures S3A–S3B).

For all three MAMPs, the reduction induced by both AP-5 and AP-7 was slightly lower in the presence of compounds modulating Ca\(^{2+}\) influx (Figures 4A–4C and Table 1, and Supplementary Figures S4C and S4D). These results suggest that, dissimilar to LPS [13], cNTP biosynthesis, and thus also probably CNGCs, do not make a major contribution to flg22-, elf18- or chitin-induced Ca\(^{2+}\) influx.

Inhibition of iGluR-like channels affects MAMP-triggered Ca\(^{2+}\) influx

The second major class of putative Ca\(^{2+}\) channels in plants comprises the iGluR-like channels, which include 20 members in Arabidopsis [9,10,19]. The present study employed various inhibitors directed against NMDA (N-methyl-D-aspartate)- 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 MAMP-triggered Δ[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 Δ[Ca\(^{2+}\)] (Figures 4A–4C and Table 1, and Supplementary Figures S4E–S4H). The observation that increased cellular Ca\(^{2+}\) concentrations in the presence of U73122 still allowed normal flg22-, elf18- and chitin-induced Δ[Ca\(^{2+}\)] (Figures 2A and 2B, and Supplementary Figures S2A and S2B) suggests that elevated steady-state Ca\(^{2+}\) levels do not themselves impede a MAMP-triggered Ca\(^{2+}\) transient. Other iGluR-channel inhibitors tested, targeting either the channel domain of NMDA-like channels (MK-801) [37] or non-NMDA iGluRs (DNQX and CNQX) [39], did not inhibit MAMP-triggered Δ[Ca\(^{2+}\)] (Table 1 and Supplementary Figure S6 at http://www.BiochemJ.org/bj/440/bj4400355add.htm). Taken together, these results provide the first evidence for a potential

### Table 1 Overview of alterations in intracellular Ca\(^{2+}\) levels upon MAMP treatment in the presence of compounds modulating Ca\(^{2+}\) transport

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Flg22</th>
<th>Elf18</th>
<th>Chitin</th>
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<td>Base</td>
<td>Peak</td>
<td>Recovery</td>
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<td>L-glutamate</td>
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measurements were started and after 2 min, flg22 (1 μM) or eliI8 (1 μM) or chitin (0.1 mg/ml) were automatically added. The luminescence originating from the seedlings was followed in time and Ca²⁺ was followed in seedlings per treatment. Each experiment was performed at least three times with similar results. Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.

Pretreatment of seedlings with both L-glutamate and L-aspartate differed largely in their physiochemical properties (Figure 4F).

Figures 3 Modulation of cNTP signalling does not influence the flg22-, eliI8- or chitin-induced Δ[Ca²⁺]

Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 10 μM U73122 (A and B), 200 μM RR (A and D), 100 μM tetracaine (E) or 100 μM nifedipine (F). The measurements were started and after 2 min, flg22 (1 μM, A, B, E and F), eliI8 (1 μM, C) or chitin (0.1 mg/ml, D) were automatically added. The luminescence originating from the seedlings was followed in time and Ca²⁺ concentrations were calculated as described previously [25]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean ± S.D. Δ[Ca²⁺] values after correction for basal Ca²⁺ levels before MAMP addition (B–F) or absolute [Ca²⁺] (A). Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.

Figure 2 No apparent contribution from internal Ca²⁺ stores and VGCCs to the flg22-, eliI8- and chitin-induced Δ[Ca²⁺]

Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 10 μM U73122 (A and B), 200 μM RR (C and D), 100 μM tetracaine (E) or 100 μM nifedipine (F). The measurements were started and after 2 min, flg22 (1 μM, A, B, E and F), eliI8 (1 μM, C) or chitin (0.1 mg/ml, D) were automatically added. The luminescence originating from the seedlings was followed in time and Ca²⁺ concentrations were calculated as described previously [25]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean ± S.D. Δ[Ca²⁺] values after correction for basal Ca²⁺ levels before MAMP addition (B–F) or absolute [Ca²⁺] (A). Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.

To further analyse the potential role for iGluR-like channels in the generation of the flg22-, eliI8- and chitin-triggered Ca²⁺ transient.

To expand the analysis of cellular responses induced by compounds that affect iGluR activity, we inspected other early MAMP-induced events. Upon biotic stress specific MAPK signalling cascades are activated, which control additional cellular responses and defence 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 statistically significant reduction (P < 0.05) of the Δ[Ca²⁺] after flg22 stimulation (Figures 4D and 4E and Table 1, and Supplementary Figures S4I and S4J). In addition, reminiscent of treatment with the inhibitors AP-5, AP-7 and kynurenic acid, basal cellular [Ca²⁺] levels were elevated to 125–158 % of the control following application of these two negatively charged amino acids (Figures 4D and 4E, and Table 1). Other amino acids did not reduce the MAMP-induced Δ[Ca²⁺] or affect basal cellular [Ca²⁺] (Figure 4F).

We then tested a range of L-glutamate concentrations for desensitization of the flg22-mediated Ca²⁺ transient and established a dose–response curve (Figure 4G). We found a concentration-dependent reduction of Δ[Ca²⁺] and a reciprocal concentration-dependent increase in steady-state Ca²⁺ levels. From a concentration of 0.2 mM L-glutamate upwards, a reduction of Δ[Ca²⁺] and an increase in basal cellular [Ca²⁺] was observed. Pretreatment with concentrations above 0.6 mM L-glutamate resulted in maximal inhibition of the flg22-triggered Ca²⁺ transient, indicating saturation of the system (Figure 4G). The concentration range where L-glutamate desensitized MAMP-induced Ca²⁺ influx is in agreement with the reported Kᵣ values for plant iGluR-like channels, which were found to be between 0.2 and 0.5 mM [41], similar to reported apoplastic amino acid concentrations (0.3–1.3 mM) [42,43]. In conclusion, the results of desensitization experiments further support a role of iGluR-like channels in MAMP-triggered Ca²⁺ influx.
between 3.5 and 5 min were averaged. Each experiment was performed at least three times with similar results. Curves shown are based on representative measurements of four to six seedlings per treatment (A–E) or the mean of n = 3 independent experiments (F). Error bars in the graphs represent the S.D. (A–E) or the S.E.M. (F–G).

(MPK6, MPK3 and MPK4) appearing 5 min after addition of L-glutamate or the MAMP stimulus (Figure 5A). Pre-incubation with either L-glutamate or kynurenic acid for 1 h resulted in low levels of MAPK activation. The subsequent addition of more L-glutamate still transiently increased activated MAPK levels. Pre-incubation of the seedlings with CNQX, which did not affect the MAMP-triggered Ca\(^{2+}\) signature, completely abolished MAPK activation after the addition of L-glutamate (Figure 5A).

Flg22, elf18 and chitin induced strong MAPK activation within 5 min after the addition of MAMP (Figures 5B and 5C, and Supplementary Figure S7 at http://www.BiochemJ.org/bj/440/bj4400355add.htm). 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 min (Supplementary Figure S7). In contrast, all three compounds markedly reduced the chitin-triggered activation of the MAPK(K/KK) cascade (Figure 5B).

Recently, Boudsocq et al. [29] showed that flg22-induced MAPK activation in Arabidopsis protoplasts does not depend, but is enhanced by, 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 LaCl\(_3\). In the case of flg22, full MAPK activation was delayed by both EGTA and LaCl\(_3\) (Supplementary Figure S7B). In the case of elf18 and chitin, EGTA reduced and LaCl\(_3\) delayed MAPK activation (Figure 5C and Supplementary Figure S7D). In conclusion, MAMP-triggered MAPK signalling requires Ca\(^{2+}\) influx for full and timely activation. Modulation of MAMP-triggered Ca\(^{2+}\) influx by iGluR inhibitors or glutamate desensitization affected MAPK activation in a similar manner as full inhibition (Figures 1A–1D) of apoplastic Ca\(^{2+}\) influx by the potent non-selective chemicals LaCl\(_3\) and EGTA.

Co-operative regulation of Ca\(^{2+}\) influx by CNGC and iGluR-like channels?

Modulating glutamate-controlled Ca\(^{2+}\) influx did largely, but not fully, abolish the MAMP-triggered Ca\(^{2+}\) transient (Figure 4, and Supplementary Figures S4 and S5). Therefore we investigated whether cNTP-regulated Ca\(^{2+}\) import was responsible for the remaining influx of Ca\(^{2+}\) and the possibility that a positive-feedback
Glutamate signalling differentially modulates MAPK- and CDPK-dependent defence gene transcript levels in response to MAMPs

To investigate how the modulation of iGluR-like channels has an impact on MAMP-triggered transcriptional activation of defence genes, we studied transcript accumulation of each MAPK- and a CDPK-dependent gene, FRK1 and PHI-1 respectively, [29] using quantitative RT-PCR. FRK1 transcript accumulation was rapidly induced (within 30 min) by each of the three MAMPs (Figure 7A). Application of either L-glutamate or kynurenic acid increased 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 (Figure 7B). Thus modulation of MAMP-triggered Ca2+ influx by either L-glutamate or kynurenic acid inhibits transcriptional up-regulation of genes downstream of the MAPK (FRK1) and CDPK (PHI-1) signalling cascades.

loop exists between both channel types. Plants were co-treated with both kynurenic acid/L-glutamate and alloxan/DDA before the addition of MAMP, and both Ca2+ levels and MAPK activation were recorded. For elf18, but not for flg22 and chitin, the combined incubations gave a stronger reduction in Δ[Ca2+] than each of the single compounds (Figure 6A and Table 1, and Supplementary Figures S8A and S8B at http://www.BiochemJ.org/bj/440/bj4400355add.htm). 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 probably represents MPK6 and MPK3, but reduced the intensity of the MAPK band with the highest electrophoretic mobility, which probably represents MPK4 (Figure 6B, and Supplementary Figures S8B and S8D). The co-inhibition of cNTP- and iGluR-mediated Ca2+ influx resulted in a combination of both effects; the major MAPK bands representing MPK3 and MPK6 showed a delayed activation (elf18) or reduced intensity (chitin) and nearly no activation of MPK4 could be detected anymore (Figure 6B, and Supplementary Figures S8B and S8D). In conclusion, we did not find evidence for a strong co-operative effect of iGluR-like channels and CNGCs in MAMP-triggered Ca2+ influx.
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 min 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 histograms. Error bars represent the S.D. of three technical replicates. The experiment was repeated three times yielding similar results.

DISCUSSION

The present study tested a comprehensive set of compounds known to target various components regulating Ca\(^{2+}\) signalling for their influence on MAMP-induced Ca\(^{2+}\) influx in Arabidopsis seedlings. Pharmacological approaches bear the risk of unspecific off-target effects, 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\(^{2+}\) channels and the expected pleiotropic phenotypes of respective mutants. We aimed to minimize the impact of off-target effects on our data by applying multiple inhibitors per target molecule class in most cases.

Inhibition of iGluR-like channels affects MAMP-triggered Ca\(^{2+}\) influx

Flg22 and elf18 induced biphasic Ca\(^{2+}\) transients that were very similar in shape and timing, whereas chitin caused a monophasic Ca\(^{2+}\) transient (e.g. Figures 1A and 1C). The presence of two separate maxima in the case of flg22 and elf18 suggests the involvement of different channel types, different subcellular Ca\(^{2+}\) stores, or a differential responsiveness between cell types. The aequorin reporter system used in the present study does not allow the detection of changes in [Ca\(^{2+}\)] at the (sub)cellular level. Therefore other reporters such as the yellow cameleon FRET (fluorescence resonance energy transfer) sensor are desirable for future analyses with improved spatial resolution [46,47].

LaCl\(_3\)-mediated inhibition of plasma membrane-resident ion channels and chelating external Ca\(^{2+}\) via EGTA treatment revealed that the primary Ca\(^{2+}\) pool for both types of MAMP-triggered Ca\(^{2+}\) signatures is the apoplast (Figures 1 and Supplementary Figure S1). This confirms and extends the findings of a previous study [45], where LaCl\(_3\) was used to demonstrate that flg22-induced Ca\(^{2+}\) influx is needed for membrane depolarization in mesophyll cells. Furthermore, both inhibition of Ca\(^{2+}\) influx by LaCl\(_3\) and chelating apoplastic Ca\(^{2+}\) were shown to prevent CDPK activation and to reduce MAPK activation in response to the peptide elicitors flg22 and elf18 in Arabidopsis protoplasts [29]. The later findings suggest a role for Ca\(^{2+}\) signalling in modulating downstream responses during MAMP-triggered immunity.

Interference with InsP\(_3\) or CICR resulted in increased steady-state Ca\(^{2+}\) levels for three of the four inhibitors tested (U73122, neomycin and RR). Notably, although basal Ca\(^{2+}\) levels were increased, a normal Ca\(^{2+}\) transient was induced (Figures 2A and 2B). In addition, RR reduced chitin- and elf18-induced Ca\(^{2+}\) transients, possibly due to its inhibitory effect on plasma membrane-resident ion channels [35,36]. The fourth compound, tetracaine, caused a slightly enhanced Ca\(^{2+}\) transient, but did not alter basal Ca\(^{2+}\) levels.

Inhibition of VGCCs did not change the intensity of MAMP-triggered Ca\(^{2+}\) transients, but two inhibitors (verapamil and nifedipine) delayed the recovery to basal cytosolic Ca\(^{2+}\) levels (Figure 2F and Supplementary Figure S3). VGCC inhibitors have documented effects in plants [35,48]. However, all three L-type channel inhibitors also have other reported targets than L-type Ca\(^{2+}\) channels, including Na\(^{+}\) channels and membrane receptors (https://www.ebi.ac.uk/chembl/db/). The prolonged Ca\(^{2+}\) 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\(^{2+}\).

Inhibitors of CNGCs shown previously to inhibit CNGC-mediated responses in planta [13], DDA and alloxan, caused only a slight reduction in the MAMP-triggered Ca\(^{2+}\) transients. Therefore in contrast with LPS [13] and the DAMP (danger-associated molecular pattern) AtPep3 [14], cNTPs, and thus probably CNGCs, do not control flg22- and elf18- and chitin-induced Ca\(^{2+}\) transient. cngc2 mutants lose the LPS-induced Ca\(^{2+}\) 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\(^{2+}\) transient is attenuated in an Atpepr1 mutant background [14], which suggests cross-talk at the level of Ca\(^{2+}\) influx between the AtPep and flg22/elf18/chitin pathways independent of cNTP generation. The delayed recovery of the MAMP-induced cytosolic rise in Ca\(^{2+}\) levels in the presence of DDA suggests a role for cNTPs in controlling Ca\(^{2+}\) 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\(^{2+}\)] and all three led to an increase in cytosolic [Ca\(^{2+}\)] (Figures 4A–4C, 4F, Table 1, and Supplementary Figures S4–S6). By contrast MK-801, CNQX and DNQX pretreatment
did not alter flg22-, elf18- or chitin-induced Ca\textsuperscript{2+} fluxes (Supplementary Figure S6). AP-5, CNQX and DNQX have been shown previously to affect iGluR-mediated processes and Ca\textsuperscript{2+} currents in pollen tube growth (AP-5, CNQX and DNQX) [21], hypocotyl elongation (DNQX) [22], glutamate-induced Ca\textsuperscript{2+} transients in mesophyll cells (CNQX and DNQX)[20] and responses to Al\textsuperscript{3+}-induced Ca\textsuperscript{2+} transients in roots (AP-5) [48]. Most recently, AP-5 was found to strongly inhibit Ca\textsuperscript{2+} influx induced by the peptide elicitor cryptogein in tobacco cell culture, whereas MK-801 and DNQX were significantly less effective in these experiments [23]. Corroborating the findings of these previous studies, we found that a subset of the iGluR inhibitors directed against both NMDA- and non-NMDA-like iGluR channels can effectively modulate MAMP-triggered Ca\textsuperscript{2+} responses in plants. In our hands, DNQX, CNQX and MK-801 did not result in inhibition of MAMP-induced Ca\textsuperscript{2+} influx (Supplementary 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\textsuperscript{2+} 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\textsuperscript{2+}, Na\textsuperscript{+} and K\textsuperscript{+} [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 the present study. Interestingly, inhibitor treatment and desensitization of the MAMP responses with L-glutamate or L-aspartate resulted in similar changes of the Ca\textsuperscript{2+} response and increased steady-state [Ca\textsuperscript{2+}] (Figure 4, and Table 1). The increased steady-state Ca\textsuperscript{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 Ca\textsuperscript{2+} channel types.

Inhibition of iGluR-like channels does not fully abolish the Ca\textsuperscript{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 Ca\textsuperscript{2+} influx would explain the biphasic shape of the Ca\textsuperscript{2+} response for these MAMPs. However, monophasic chitin-induced fluxes are altered in a similar manner, which argues for an alternative explanation of the phenomenon. The present study suggests that the potential second channel class of channels is not cNTP-activated, since co-treatment with inhibitors of adenylyl cyclases and iGluR-like channels did not fully block Ca\textsuperscript{2+} influx (Figure 6A).

We exploited the phenomenon of iGluR channel desensitization by analysing the MAMP response after pretreatment with various amino acids. In the present study, flg22-, elf18- and chitin-induced responses were only desensitized by the negatively charged L-glutamate and L-aspartate (Figures 4D–4G, and Supplementary Figures S4I and S1J). 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 the present study 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 modelling of the glutamate-binding site of plant iGluR-like channels suggested that only AtGLR1.1 can bind glutamate residues, whereas the other family members bind glycine [22]. This suggests that the iGluR complex responsible for MAMP-triggered Ca\textsuperscript{2+} influx would have to contain AtGLR1.1. However, modelling 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 Ca\textsuperscript{2+} influx controls defence gene expression

Reduction of MAMP-triggered Ca\textsuperscript{2+} influx fully inhibited induced 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 Supplementary Figure S7) the strong impact on MAPK-dependent FRK1 expression was surprising. This result suggests that either the signal transduction cascade leading from Ca\textsuperscript{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 Ca\textsuperscript{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 conclusion, these results indicate a crucial role for cellular signalling events upon MAMP perception that rely on iGluR-mediated Ca\textsuperscript{2+} influx. It remains to be seen to what extent the reduction in defence gene activation translates into a compromised immune response. In this context it would be highly desirable to examine the effect of interference with Ca\textsuperscript{2+} signalling during authentic plant–microbe interactions. However, the presumed effect of compounds on both the host and the parasite complicates the interpretation of data from such experiments emphasize the limitations of pharmacological approaches.

### A model for iGluR-mediated Ca\textsuperscript{2+} influx

On the basis of the results of the present study we propose the following model for MAMP-triggered Ca\textsuperscript{2+} influx (Figure 8). Following MAMP perception by cognate PRRs, glutamate-dependent opening of iGlu-like channels and Ca\textsuperscript{2+} influx from
the apoplast into the cytoplasm is activated by as yet unknown mechanisms. This may involve vesicle-mediated secretion of L-glutamate into the apoplast [23]. Elevated cytoplasmic Ca\(^{2+}\) levels translate via CDPK- and MAPK-dependent pathways into defence gene activation. It remains unclear how Ca\(^{2+}\) influx via iGluR-like channels is terminated. One possibility is a negative-feedback loop that involves Ca\(^{2+}\)-dependent CaM (calmodulin) 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 this study at a different experimental level. The analysis of a subset of Arabidopsis iGluR single mutants did not yet result in the identification of mutants that exhibit altered MAMP-induced Ca\(^{2+}\) signatures (results not shown). These preliminary data indeed argue for redundant iGluR functions in plant innate immunity.

**AUTHOR CONTRIBUTION**

Mark Kwaaitaal and Ralph Panstruga designed the research; Mark Kwaaitaal, Rik Huisman, Jens Maintz and Anja Reinstätler performed the experiments; Mark Kwaaitaal and Ralph Panstruga analysed the data; and Mark Kwaaitaal and Ralph Panstruga wrote the paper.

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SUPPLEMENTARY ONLINE DATA

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

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Figure S1 Flg22, elf18 and chitin induce Ca²⁺ influx from the apoplast

Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 10 mM EGTA (A and B). The measurements were started and after 2 min elf18 (1 μM; A) or chitin (0.1 mg/ml; B) were automatically added. The luminescence originating from the seedlings was followed in time and Ca²⁺ concentrations were calculated as described previously [1]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean ± S.D. Δ[Ca²⁺] values after correction for basal Ca²⁺ levels before MAMP addition (A and B). Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.

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Figure S2  No apparent contribution from internal Ca^{2+} stores to the flg22-, elf18- and chitin-induced Δ[Ca^{2+}]

Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 10 μM U73122 (A and B), 50 μM neomycin (C–F), 200 μM RR (G and H) or 100 μM tetracaine (I and J). The measurements were started and after 2 min, flg22 (1 μM; A, C, D, G and H), elf18 (1 μM; A, E and I) or chitin (0.1 mg/ml; B, F and J) were automatically added. The luminescence originating from the seedlings was followed in time and Ca^{2+} concentrations were calculated as described previously [1]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPS. Shown are mean ± S.D. Δ[Ca^{2+}] values after correction for basal Ca^{2+} levels before MAMP addition (A, B, D–F and H–J) or absolute [Ca^{2+}] (G and H). Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.
Figure S3  No apparent contribution from VGCCs to the flg22-, elf18- and chitin-induced Δ[Ca²⁺] 

Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 10 μM nifedipine (A and B), 100 μM verapamil (C–E) or 100 μM diltiazem (F–H). The measurements were started and after 2 min, flg22 (1 μM; C and F), elf18 (1 μM; A, D and G) or chitin (0.1 mg/ml; B, E and H) were automatically added. The luminescence originating from the seedlings was followed in time and Ca²⁺ concentrations were calculated as described previously [1]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean ± S.D. Δ[Ca²⁺] values after correction for basal Ca²⁺ levels before MAMP addition. Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.
Modulation of cNTP signalling has no influence, but modulation of iGluR-like channel function reduces the flg22-, elf18- or chitin-induced $\Delta [Ca^{2+}]$.

Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 1 mM alloxan (A and B), 200 $\mu$M DDA (C and D), 1 mM AP-5 (E and F), 1 mM kynurenic acid (G and H) or 1 mM L-glutamate (I and J). The measurements were started and after 2 min, elf18 (1 $\mu$M, A, C, E, G and I) or chitin (0.1 mg/ml, B, D, F, H and J) was automatically added. The luminescence originating from the seedlings was followed in time and $Ca^{2+}$ concentrations were calculated as described previously (1). The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean $\pm$ S.D. $\Delta [Ca^{2+}]$ values after correction for basal $Ca^{2+}$ levels before MAMP addition. Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.

Figure S4  Modulation of cNTP signalling has no influence, but modulation of iGluR-like channel function reduces the flg22-, elf18- or chitin-induced $\Delta [Ca^{2+}]$.
iGluR-like channels mediate MAMP-triggered calcium influx in Arabidopsis

Figure S5 Reproducible reduction of the flg22-, elf18- or chitin-induced $\Delta [\text{Ca}^{2+}]$ following iGluR inhibition

Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 1 mM L-glutamate (A, D and G), 1 mM kynurenic acid (A, D and G), 1 mM AP-5 (B, E and H), 1 mM alloxan (C, F and I) or 200 $\mu$M DDA (C, F and I). The measurements were started and after 2 min, flg22 (1 $\mu$M; A–C), elf18 (1 $\mu$M; D–F) or chitin (0.1 mg/ml; G–I) was automatically added. The luminescence originating from the seedlings was followed in time and Ca$^{2+}$ concentrations were calculated as described previously [1]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean $\pm$ S.E.M. $\Delta [\text{Ca}^{2+}]$ values of four or more biological replicates after correction for basal Ca$^{2+}$ levels before MAMP addition.
Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated for 1 h with 100 μM MK-801 (A–C), 1 mM DNXQ (D–F) or 1 mM CNQX (G–I). The measurements were started and after 2 min, flg22 (1 μM; A, D and G), elf18 (1 μM; B, E and H) or chitin (0.1 mg/ml; C, F and I) was automatically added. The luminescence originating from the seedlings was followed in time and Ca\(^{2+}\) concentrations were calculated as described previously [1]. The grey marking delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean ± S.D. ∆[Ca\(^{2+}\)] values after correction for basal Ca\(^{2+}\) levels before MAMP addition. Curves shown are based on representative measurements of four to six seedlings per treatment. Each experiment was performed at least three times with similar results.
Figure S7  Modulation of iGluR-like channel function or inhibition of Ca\(^{2+}\) influx from the apoplast affects MAPK activation

Aequorin-expressing Arabidopsis seedlings (10–12-day-old) were pretreated with 1 h L-glutamate (L-glu) (A–D), 1 mM kynurenic acid (KA) (A and C), 1 mM CNQX (A and C), 1 mM LaCl\(_3\) (B and D) or 10 mM EGTA (B and D). Time point 0 minutes was harvested before MAMP addition. Then 1 \(\mu\)M flg22 (A and B) or 1 \(\mu\)M elf18 (C and D) was added and after 5 min and 15 min samples were taken and analysed for MAPK activation as described previously [2]. Per sample 10–12 seedlings were used. Arrows mark the activated MAPKs on the blots. Experiments were performed two or three times with similar results. Molecular mass is given in kDa on the left-hand side. The lower panel of each blot shows the staining of the membrane with Ponceau S to visualize equal loading.
Figure S8  iGluR signalling and cNTP signalling act additively in elf18-induced Ca\textsuperscript{2+} influx, but not in fg22- or chitin-induced Ca\textsuperscript{2+} influx

Aequorin-expressing Arabidopsis seedlings (A and C) and Col-0 wild-type seedlings (B and D) (10–12-day-old) were pretreated for 1 h with 1 mM l-glutamate (L-glu), 1 mM alloxan or a combination of both. (A and C) Aequorin-based Ca\textsuperscript{2+} measurements: 2 min after initiation of luminescence detection, flg22 (1 μM; A) or chitin (0.1 mg/ml; C) was automatically added. The luminescence originating from the seedlings was followed in time and Ca\textsuperscript{2+} concentrations were calculated as described previously [1]. The grey marking, delineates the spike in luminescence caused by the injection of the MAMPs. Shown are mean ± S.D. Δ[Ca\textsuperscript{2+}] of >three biological replicates per treatment after correction for base Ca\textsuperscript{2+} levels with the standard error of mean. (B and D) MAPK assays: time point 0 min was harvested before MAMP addition for the MAPK assays. Then 1 μM flg22 (B) or 0.1 mg/ml chitin (D) was added and after 5 min and 15 min samples were taken and analysed for MAPK activation as described previously [2]. Per sample 10–12 seedlings were used. Molecular mass is given in kDa on the left-hand side.

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