

On the ligand binding profile and desensitization of plant ionotropic glutamate receptor (iGluR)-like channels functioning in MAMP-triggered Ca²⁺ influx

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Abbreviations: AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid; CaM, calmodulin; CaMBD, calmodulin binding domain; CHX, cycloheximide; iGluR, ionotropic glutamate receptor; MAMP, microbe-associated molecular pattern; NMDA, *N*-methyl-D-aspartic acid; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

The generation of intracellular microbe-associated molecular pattern (MAMP)-triggered Ca²⁺ transients was recently demonstrated to involve ionotropic Glutamate Receptor (iGluR)-like channels in *Arabidopsis* and tobacco. Here we elaborate on our previous findings and refine our insights in the putative agonist binding profile and potential mode of desensitization of MAMP-activated plant iGluRs. Based on results from pharmacological inhibition and desensitization experiments, we propose that plant iGluRs complexes responsible for the MAMP-triggered Ca²⁺ signature have a binding profile that combines the specificities of mammalian NMDA- and non-NMDA types of iGluRs, possibly reflecting the evolutionary history of plant and animal iGluR. We further hypothesize that, analogous to the mammalian NMDA-NR1 receptor, desensitization of plant iGluR-like channels might involve binding of the ubiquitous Ca²⁺ sensor calmodulin to a cytoplasmic C-terminal domain.

iGluR-Like Channel Function in Plant Defense and Development

Ionotropic Glutamate Receptor (iGluR)-like channels represent a family of integral membrane proteins that control amino acid-gated cation fluxes. This protein class is best known for its key role in vertebrate neurotransmission.¹ Recently, two groups demonstrated the involvement of iGluR-like channels in microbe-associated molecular pattern (MAMP)-triggered Ca²⁺ fluxes in plants. In the presence of iGluR channel agonists and antagonists, the MAMP-induced Ca²⁺ transient was considerably reduced and downstream responses were attenuated both in tobacco (*Nicotiana tabacum*) cell cultures in response to cryptogein² and intact *Arabidopsis thaliana* seedlings in response to flg22, elf18 and chitin, respectively.³ These altered responses include mitogen-activated protein kinase (MAPK) activation, transcriptional regulation of downstream response genes³ and nitric oxide production.²

In addition, cryptogein induces exocytosis-dependent accumulation of glutamate in the apoplastic space.² Being present as a family with 20 members in *Arabidopsis*,⁴ the acquisition of genetic evidence corroborating the role of these channels in MAMP-triggered Ca²⁺ influx is cumbersome and will require extensive future work. These receptors likely function as heterotypic quaternary complexes, therefore the members might compensate for each other's function in the complex. Results from desensitization experiments, using several amino acids in *glr3.3* and *glr3.4* single or double mutants, suggested that multiple subunits together control amino acid-dependent Ca²⁺ influx. These data fit a model where various quaternary complexes of iGluR-like channels with dissimilar sensitivity to different amino acids co-exist.⁵

Mutants in some *iGluR* family members, either as single knockout or mutant combinations, show altered Ca²⁺ responses or developmental phenotypes. For example, *glr3.3* mutants exhibit more variable root growth compared with wild type plants upon a

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gravity stimulus⁶ and *glr1.2* and *glr3.7* mutants have altered Ca²⁺ responses in pollen tubes and altered pollen tube morphology and growth speed, respectively.⁷ Overexpression of *AtGLUR2/GLR3.1* alters Ca²⁺ homeostasis and utilization and renders plants sensitive to ion stress.⁸ Moreover, *GLR1.1* is involved in stomatal closure and abscisic acid signaling and contributes to fungal disease resistance.^{9,10}

Ligand Preference of Arabidopsis iGluRs Contributing to MAMP-Triggered Ca²⁺ Transients

In mammalian systems, three main subclasses of iGluR-like channels with ion gating activity are distinguished, partly based on their selective preference for synthetic/non-native agonists: the NMDA (*N*-Methyl-D-aspartic acid), the AMPA (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid) and the kainate-binding receptor channels. The AMPA and kainate receptor channels are generally grouped together as the non-NMDA class.¹¹⁻¹³ In the native situation, mammalian NMDA receptor channels are activated by L-aspartate and L-glutamate and additionally require binding of the co-agonists D-serine or glycine at an allosteric binding site for activity.^{1,11,13-15}

We studied if the plant repertoire of iGluR channels found to be involved in MAMP-triggered Ca²⁺ influx shows any preference toward these synthetic agonists using the desensitization assays recently described by Kwaaitaal and coworkers.³ Pretreatment of transgenic Arabidopsis seedlings expressing the Ca²⁺ sensor aequorin with the agonists prior to MAMP addition revealed that NMDA, like L-glutamate and L-aspartate, strongly inhibits the flg22-induced Ca²⁺ response and increases steady-state Ca²⁺ levels (see ref. 3, **Fig. 1A and B**). Using the phospholipase C inhibitor U-73122 as precedence, we previously showed that an increase in steady-state [Ca²⁺] does not per se inhibit a normal MAMP-triggered Ca²⁺ signature.³ The non-NMDA agonists kainic acid and AMPA also reduced the flg22-induced Ca²⁺ transient and increased steady-state Ca²⁺ levels, but to a lesser extent than NMDA (**Fig. 1C and D**). By contrast, pre-treatment with the NMDA receptor co-agonist D-serine did not alter Ca²⁺ responses to flg22 (**Fig. 1A and B**). In sum, these results corroborate our and others' previous findings indicating a role for iGluR-like channels in the generation of MAMP-induced Ca²⁺ transients.^{2,3} In addition, the data suggest that plant iGluR-like channel complexes have essentially a ligand binding profile that combines the specificities of both major classes of mammalian iGluR-like channels, the NMDA and the non-NMDA receptor channels. However, the effect of NMDA on the activity of Arabidopsis iGluRs seems to be more pronounced, suggesting a tendency toward a stronger responsiveness of the Arabidopsis iGluR complexes to NMDA-like agonists. This interpretation is further supported by previous data revealing that the NMDA receptor antagonists AP-5 and AP-7 cause a strong decrease in MAMP-induced Ca²⁺ transients, while the non-NMDA receptor antagonists CNQX and DNQX showed either no or a much weaker effect.^{2,3,7} Our pharmacological classification of plant iGluRs according to their responsiveness to various inhibitors is consistent with the phylogenetic analysis of plant *iGluR* genes,

in which plant iGluRs grouped separately from mammalian NMDA and non-NMDA type iGluRs and which indicated that the divergence of animal and plant *iGluR* genes likely predated the divergence of iGluR subtypes in animals.^{4,11}

Control of iGluR Desensitization

After ligand binding and pore opening, iGluR-like channels are temporarily desensitized for a new response. This feature of individual iGluRs, together with Ca²⁺ efflux from the cytosol mediated by Ca²⁺-ATPases and anion exchange channels,¹⁶ may contribute to shaping the integrated Ca²⁺ signature seen in experimental systems using the luminescent aequorin Ca²⁺ sensor. Several molecular modes of transient iGluR inactivation have been described, including structural rearrangements of the membrane-spanning and/or extracellular domains as well as binding of the Ca²⁺ sensor calmodulin (CaM) to the cytosolic C-terminus of the receptor channel.^{12,17-20} Precedence for the latter is provided by the mammalian *N*-methyl-D-aspartate receptors (NMDARs), a class of iGluR-like channels that are major contributors to Ca²⁺ flux into brain neurons and that play a critical role in learning, memory, neural development and synaptic plasticity. NMDARs are composed of two families of subunits, termed NR1 and NR2. Upon Ca²⁺ influx, the C-terminal tail of NR1 subunits binds CaM to temporarily inactivate channel function and possibly to regulate receptor trafficking.^{17,20-22}

In the presence of Ca²⁺, CaM binding stimulates the formation of an amphipathic α -helix in otherwise largely unstructured regions of target proteins. The hydrophobic face of the helix then interacts with a hydrophobic pocket of Ca²⁺-CaM. For its interaction with the α -helix, Ca²⁺-CaM requires a series of properly spaced basic and hydrophobic residues for binding. From the analysis of a large number of CaM binding domains (CaMBDs), several classes of consensus sequences were deduced. Examples are the IQ class, the 1-8-14 and the 1-5-10 classes, where the numbers indicate the position of bulky hydrophobic residues that are needed for Ca²⁺-CaM binding.^{23,24}

We examined the sequences of the 20 Arabidopsis iGluRs in silico for the presence of potential CaMBDs. In analogy to the location of the CaMBD in mammalian NMDA NR1 subunits, we focused our analysis on the C-terminal intracellular domain (**Fig. 2 and Fig. S1**). We found that nine of the 20 Arabidopsis iGluRs possess a putative CaMBD in this region (**Fig. 2**). Notably, supposed CaMBDs were found in all iGluR subfamilies, suggesting that their presence might be a general feature of plant iGluR-like channels. However, for some iGluRs this region overlaps with the prediction of the most C-terminal transmembrane domain; in these instances the relevance of the CaMBD prediction is questionable. Absence of predicted CaMBDs in approximately half of the Arabidopsis iGluRs may either result from false-negative predictions or might indicate isoform-specific differences in the mode of receptor desensitization.

Additional putative CaMBDs were predicted in the intracellular loop between the pore region (TM2) and TM3 and in the large extracellular domain between TM3 and TM4 (**Fig. S1**). The stretch of amino acids between TM2 and TM3 is relatively

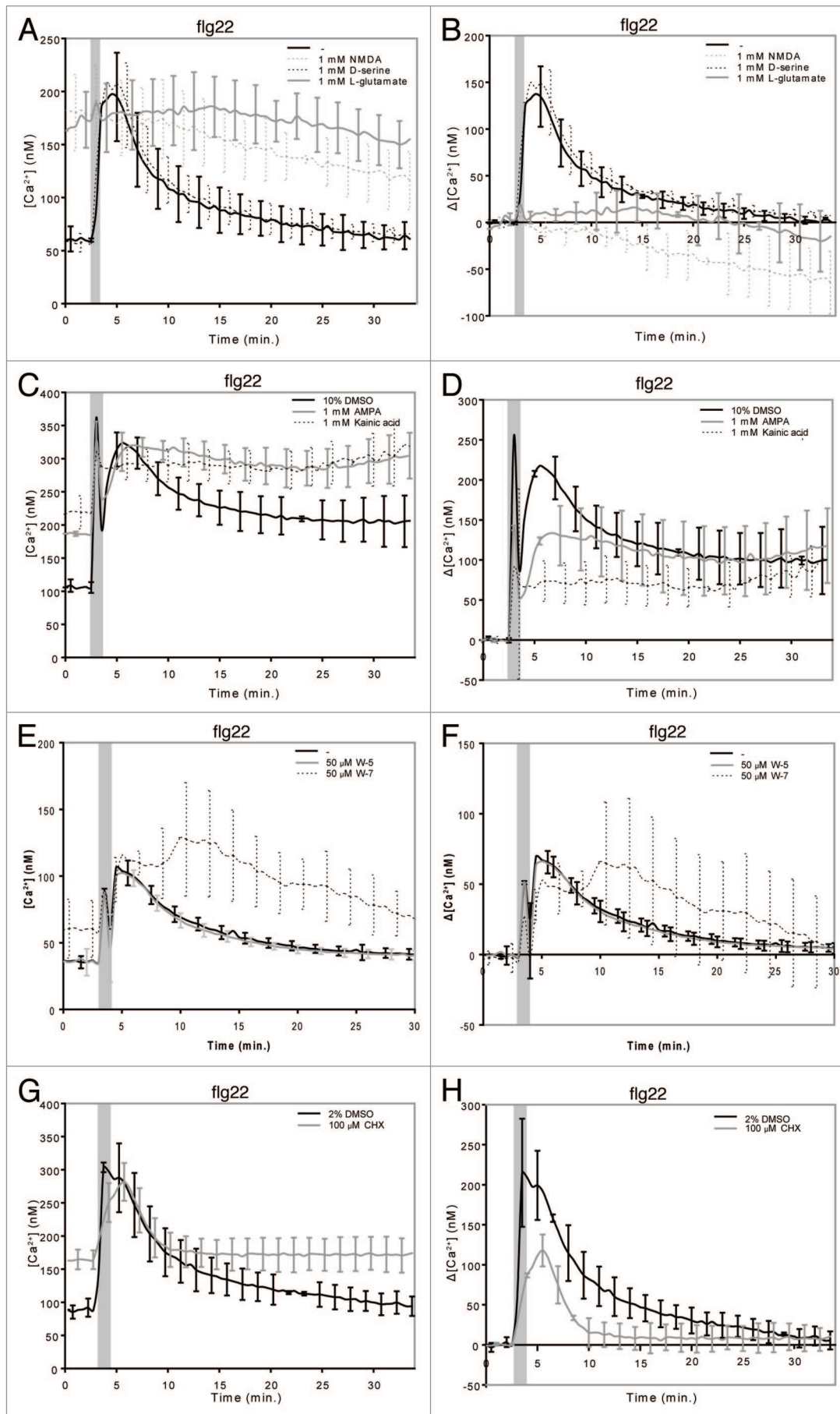


Figure 1. (See page 1375.) Ligand specificity of MAMP-stimulated iGluRs and molecular control of iGluR desensitization. 10-12-d-old seedlings were pre-treated for 30 min with (co-) agonists, W-7 or CHX as described in Kwaaitaal et al. (2011) and then 1 μ M flg22 was added to trigger a Ca²⁺ transient. Seedlings were pre-treated with (A, B) 1 mM NMDA, 1 mM D-serine, 1 mM L-glutamate, (C, D) 1 mM AMPA, 1 mM kainic acid, (E, F) 50 μ M W-5 or W-7 or (G, H) 100 μ M CHX. Shown are the absolute Ca²⁺ concentrations (A, C, E and G) or the background-corrected differences in cellular Ca²⁺ levels (B, D, F and H). Data given are the mean \pm standard deviation of 6–12 seedlings per measurement and represent typical examples of three or more independent experiments. Note that the first short and strong Ca²⁺ peak marked by the gray bar results from the mechanical stimulation caused by the addition of the stimulus. Also note the large standard deviation of the W-7-treated seedlings compared with the control measurements, which reflects the high variation in the individual seedling curves upon treatment with this compound (see Fig. S2). For further experimental details see reference 3.

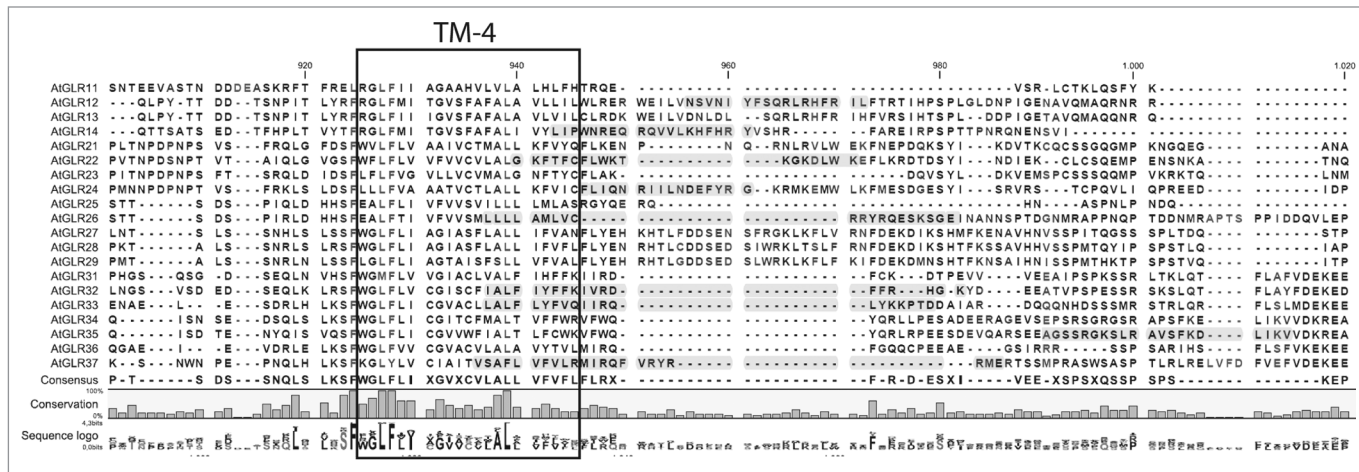


Figure 2. Presence of a potential CaMBD at the cytoplasmic C-terminus of Arabidopsis iGluRs. Transmembrane domain prediction was performed using the Aramemnon (<http://aramemnon.botanik.uni-koeln.de/>) website and the stretches predicted using a hidden Markov model²⁹ are marked. Putative CaMBDs (marked in gray) were identified based on the following web tool: <http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html>.²³ For the prediction the last 268–375 amino acids were used, starting from the first TM domain and excluding the extracellular N-terminal domain. Marked residues represent those having a score higher than six. Alignments were generated in the CLC Workbench package (www.clcbio.com).

short and is localized between two membrane-embedded regions. This likely limits its mobility and probably hampers the formation of the amphipathic α -helix needed for Ca²⁺-CaM binding. Binding of CaM to extracellular binding sites has been reported and application of external CaM induces Ca²⁺ influx in plants.²⁵ Although potentially exciting, control of mammalian NMDARs by extracellular CaM binding has to our knowledge not been observed or studied, making the validity of these putative extracellular CaMBDs unlikely. In addition, the extracellular CaMBD predicted in GLR2.9 includes an amino acid (E) potentially involved in ligand binding.¹⁴ This observation suggests that either also other features match the consensus sequences for CaMBDs or that the two domains overlap. Taken together, the predicted C-terminal regions remain the most likely CaMBD candidates in the Arabidopsis iGluR family.

To experimentally assess a potential role for CaM in controlling iGluR-mediated Ca²⁺ fluxes we pre-treated aequorin-expressing Arabidopsis seedlings with the well-characterized CaM binding inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7)²⁶ or its inactive analog *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5).^{27,28} Upon W-7 application we found an increased steady-state [Ca²⁺] (Fig. 1E), a slight inhibition of the Ca²⁺ transient in response to flg22 together with a broadening of the Ca²⁺ peak and an irregular recovery especially noticeable in individual seedling response

curves (Fig. 1E and F, Fig. S2A and B). By contrast, W-5-treated seedlings behaved like water-treated controls (Fig. 1E and F, Fig. S2A and B). These observations suggest a direct or indirect CaM-dependent control of iGluR channel gating or closure. The broadening of the Ca²⁺ peak plus the irregular recovery could indicate that W-7 also interferes with CaM binding to other channel classes (e.g., cyclic nucleotide-gated channels) or affects Ca²⁺ efflux from the cytosol mediated by Ca²⁺-ATPases or anion exchange channels.¹⁶

In a complementary experiment we used the protein biosynthesis inhibitor cycloheximide (CHX). We observed an increase in steady-state Ca²⁺ levels following such a pre-treatment that was higher than after W-7 treatment, similar in magnitude as the one observed upon treatment with NMDA or L-glutamate, but lower in magnitude than the one observed following treatment with AMPA or kainate (Fig. 1G; compare with Fig. 1A, C, E and G). In addition, the MAMP-induced Ca²⁺ transient was lower in magnitude, but had a similar shape as the curve of untreated seedlings (Fig. 1H). These phenomena suggest that a class of proteins with a short half-life control the activity of Ca²⁺ in- or efflux channels in plants. Given the similarities in the effect of W-7 and CHX treatments on the flg22-triggered Ca²⁺ signature (Fig. 1E–H) one may speculate that inhibition of protein biosynthesis with CHX could affect cellular CaM levels. The increase in cytosolic [Ca²⁺] upon CHX treatment might thus

reflect the loss of CaM-mediated control of Ca²⁺ channels and Ca²⁺ ATPases, thereby allowing more Ca²⁺ influx and/or preventing efficient Ca²⁺ efflux, respectively.

In conclusion, the prediction of CaMBDs in a subset of the Arabidopsis iGluR-like channels plus the altered MAMP-triggered Ca²⁺ responses in the presence of W-7 point toward a role for CaM binding in plant iGluR control. To confirm that CaM binding indeed regulates iGluR activity, experiments testing the binding of CaM to iGluR-like channels are desired. The analysis of iGluR mutant variants that either lack the proposed CaMBD or harbour defective versions thereof promise to provide additional insights.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here: <http://www.landesbioscience.com/journals/psb/article/21761/>

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