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REVIEW PAPER

Comparing plant and animal glutamate receptors: common traits but different fates?

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Abstract

Animal ionotropic glutamate receptors (iGluRs) are ligand-gated channels whose evolution is intimately linked to that of the nervous system, where the agonist glutamate and co-agonists glycine/D-serine act as neurotransmitters or neuromodulators. While iGluRs are specialized in neuronal communication, plant glutamate receptor-like (GLR) homologs have evolved many plant-specific physiological functions, such as sperm signaling in moss, pollen tube growth, root meristem proliferation, innate immune, and wound responses. GLRs have been associated with Ca^{2+} signaling by directly channeling its extracellular influx into the cytosol. Nevertheless, very limited information on functional properties of GLRs is available, and we mostly rely on structure/function data obtained for animal iGluRs to interpret experimental results obtained for plant GLRs. Yet, a deeper characterization and better understanding of plant GLRs is progressively unveiling original and different functions when compared with their mammalian counterparts. Here, we review the function of plant GLRs comparing their predicted structure and physiological roles with those of the well-documented roles of iGluRs. We conclude that interpreting GLR function based on comparison with their animal counterparts calls for caution, especially when presuming physiological roles and the mode of action for plant GLRs, and when comparing iGluRs in neuronal tissues with those in peripheral, non-neuronal tissues.

Keywords: Ca^{2+} signaling, cation channel, electric signaling, GLR, glutamate receptor-like channel, iGluR, ionotropic glutamate receptor, structure–function.

Introduction

Ionotropic glutamate receptors (iGluRs) have been identified in all three domains of life, and their presence in the animal branch ranges from ctenophores to vertebrates. Homologs of iGluRs, known as glutamate receptor-like (GLR) channels, were also found in the genomes of *Chlamydomonas*, chlorophytes, mosses, ferns, gymnosperms, and flowering plants (De Bortoli *et al.*, 2016) (Fig. 1). It is noteworthy that GLRs are absent from yeast, eubacteria, archaebacteria, and fungi (Chiu *et al.*, 1999), suggesting kingdom-specific roles for iGluRs and GLRs in animals and plants, respectively. Indeed, in mammals, iGluRs play an important role in integrative cognitive processes such as memory and learning, and they have been linked to the pathology of depression and psychosis as well as neurodegenerative diseases, including Alzheimer's disease (Reinders *et al.*, 2016). The evolution of iGluRs is intimately linked to central nervous system complexification (Jorgensen, 2014). Remarkably, iGluRs are believed to be fundamental for neuronal signaling, development, and plasticity in phyla as ancient as cnidarians, such as hydra (Pierobon, 2012), and are conserved in the more evolved phyla of nematodes and vertebrates (Fig. 1).

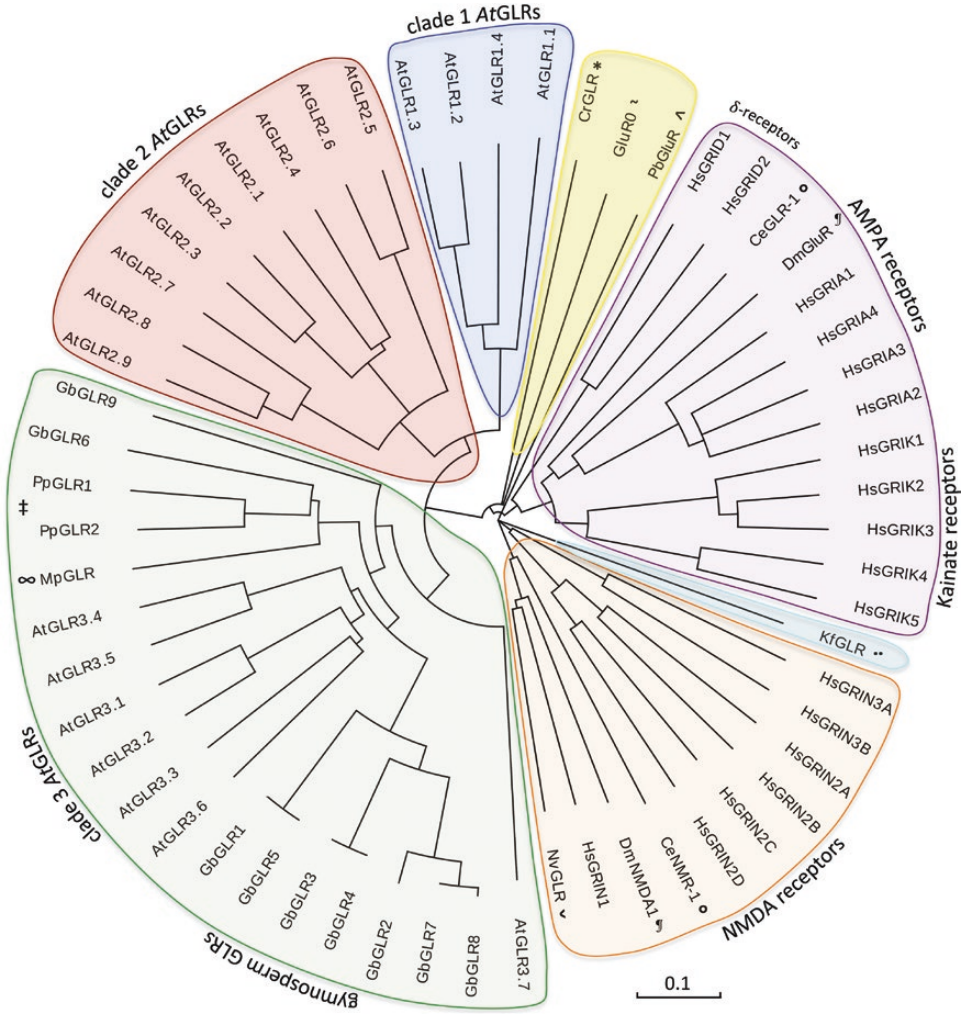

Fig. 1. Phylogram of selected glutamate receptors from bacteria, plants, and animals. The tree shows the phylogenetic relationship of chosen glutamate receptors from *Arabidopsis thaliana* (*At*), the nematode *Caenorhabditis elegans* (*Ce*, °), the unicellular green alga *Chlamydomonas rheinhardtii* (*Cr*, *), the fruit fly *Drosophila melanogaster* (Dm, ¶), the gymnosperm *Ginkgo biloba* (*Gb*), *Homo sapiens* (*Hs*), the filamentous green alga *Klebsormidium flaccidum* (*Kf,* ¨), the liverwort *Marchantia polymorpha* (*Mp,* ∞), the sea anemone *Nematostella vectensis* (*Nv,* ˇ), the moss *Physcomitrella patens* (*Pp,* ‡), the ctenophore *Pleurobrachia bachei* (*Pb,* ^), and the cyanobacterium *Synechocystis* sp. (GluR0, ˜). The tree was generated with the Clustal Omega Neighbor–Joining method software (Sievers *et al.*, 2011). The scale bar indicates substitutions per site. Sequences used can be found in Supplementary Fig. S1 at *JXB* online. (This figure is available in colour at *JXB* online.)

Plants are characterized by the absence of a nervous system or any structure allowing for neuron-like electric signaling, and thus the discovery of plant GLRs was an unexpected revelation (Lam *et al.*, 1998). Since then, and contrary to their overly specialized animal counterparts, plant GLRs have been shown to be implicated in a vast variety of cellular processes and different aspects of plant and cell physiology. For instance, GLRs have been linked to carbon and nitrogen metabolism (Kang and Turano, 2003; Kang *et al.*, 2004), abscisic acid (ABA) biosynthesis and signaling (Kang and Turano, 2003; Kang *et al.*, 2004; Kong *et al.*, 2015), water loss (Kang *et al.*, 2004; Lu *et al.*, 2014), root gravitropism (Miller *et al.*, 2010), lateral root initiation (Vincill *et al.*, 2013), root development (Singh *et al.*, 2016), innate immune responses (Kang *et al.*, 2004; Kwaaitaal *et al.*, 2011; Li *et al.*, 2013; Manzoor *et al.*, 2013; Forde and Roberts, 2014), stomatal closure (Cho *et al.*, 2009), pollen tube growth (Michard *et al.*, 2011; Wudick *et al.*, 2018), self-incompatibility (Iwano *et al.*, 2015), wound-induced leaf to leaf electric signaling (Mousavi *et al.*, 2013), seed germination (Kong *et al.*, 2015), response to aphid feeding (Vincent *et al.*, 2017), and moss sperm signaling (Ortiz-Ramírez *et al.*, 2017).

Despite the broad physiological relevance of GLRs, there is no clear understanding about many of their fundamental regulatory aspects, namely concerning their endogenous ligand(s), gating, their ion selectivity, or the subcellular localization of most members. Exceptions for some of these aspects include *At*GLR1.4 and 3.4 and the moss *Pp*GLR1, which were shown to have a poor ionic selectivity when heterologously expressed in mammalian cells or *Xenopus* oocytes (Vincill *et al.*, 2012; Tapken *et al.*, 2013; Ortiz-Ramírez *et al.*, 2017). Although initially expected to be plasma membrane channels, some plant GLRs were subsequently detected in chloroplasts and mitochondria (Teardo *et al.*, 2011, 2015), as well as in the sperm cell (endo)membranes and the vacuolar membrane (Table 1) (Wudick *et al.*, 2018), suggesting a diversification of their localization in plants. The lack of any

precise functional characterization of GLRs makes it impossible to understand the cellular and molecular mechanisms behind the growing number of phenotypes described in the literature. In the absence of plant-specific data regarding the structure, function, and regulation of GLRs, results are often—and understandably—interpreted in the context of and compared with animal iGluRs. Excellent reviews with a focus on GLR function and phylogeny have recently been published (e.g. Price *et al.*, 2012; Forde and Roberts, 2014; De Bortoli *et al.*, 2016; Weiland *et al.*, 2016), along with original data demonstrating some unique features of early land plant GLRs (Ortiz-Ramírez *et al.*, 2017). In this review, we place an emphasis on the emerging differences between iGluRs and GLRs. Focusing on the scarce functional data available for plant GLRs and comparing GLR sequences, their predicted structures and functional domains with the respective parts from iGluRs, we want to raise awareness of obvious differences between both. These comparisons reflect the profoundly different evolutionary path taken by plants and animals, and are informative of the major specialization and functionalization steps that took place during the development of different traits and adaptations. Ultimately, we aim at challenging the interpretation of plant GLR function based on findings from iGluRs when taking first principles into consideration to explain the available data.

Table 1. *Prediction of N-terminal transmembrane domain (N-TMD) and documented localization*

*At*GLR	Predicted N-TMD?		Plant localization
*At*GLR1.1	7/18	++	
*At*GLR1.2	13/18	+++	
*At*GLR1.3	9/18	++	
*At*GLR1.4	15/18	+++	P[a]
*At*GLR2.1	14/18	+++	V[b]
*At*GLR2.2	6/18	+	
*At*GLR2.3	8/18	++	
*At*GLR2.4	13/18	+++	
*At*GLR2.5	4/18	+	
*At*GLR2.6	13/18	+++	
*At*GLR2.7	12/18	++	
*At*GLR2.8	7/18	++	
*At*GLR2.9	9/18	++	
*At*GLR3.1	17/18	+++	
*At*GLR3.2	9/18	++	P[c]
*At*GLR3.3	5/18	+	P[c], S[b]
*At*GLR3.4	9/18	++	C, P[c–f]
*At*GLR3.5.1	3/18	+	M[g]
*At*GLR3.5.2	1/18	+	C[g]
*At*GLR3.5.3	6/12	+	
*At*GLR3.6	7/18	++	
*At*GLR3.7	11/18	++	

Predicted likelihood of bearing an N-TMD: high (+++), medium (++), low (+), based on results from 18 different prediction programs (aramemnon.uni-koeln.de). Multiple *At*GLR3.5 splicing variants were included due to their differential localization. C, chloroplast; M, mitochondrion, P, plasma membrane; S, sperm cell; V, vacuole; [a] Tapken *et al.* (2013); [b] Wudick *et al.* (2018); [c] Vincill *et al.* (2013); [d] Meyerhoff *et al.* (2005); [e] Teardo *et al.* (2011); [f] Vincill *et al.* (2012); [g]Teardo *et al.* (2015).

The ontology of the animal iGluR and plant GLR family

Glutamate receptors are part of the superfamily of gated channels, which include the eubacterial proton-gated K^+ channel KscA, and derivative eukaryotic voltage- and ligand-gated channels (Wo and Oswald, 1995; Price *et al.*, 2012). In mammals, four iGluR clades have been differentiated based on their divergent sequences and ligand specificities (Traynelis *et al.*, 2010) (Fig. 1). The most ancestral clade, present as early as in cnidarian species, consists of *N*-methyl-D-aspartate (NMDA) receptors that differentiated into α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Chiu *et al.*, 1999; Price *et al.*, 2012; De Bortoli *et al.*, 2016; Greer *et al.*, 2017). A fourth group is comprised of two delta receptors (δ-receptors), which share sequence and structural homology with iGluRs, but cannot be activated by glutamate. In addition to different ligand affinities, the members from each iGluR clade also differ by their (de) activation kinetics, as well as their ionic selectivities (Traynelis *et al.*, 2010), giving to each group of iGluRs a specific role in the highly complex and evolved nervous system.

Based on their sequence homology and the analyzed sequence region, plant GLRs were shown to be similar to both NMDA and non-NMDA receptor-like iGluRs, suggesting a divergence of iGluRs/GLRs prior to their clade differentiation (Weiland *et al.*, 2016). Sequence alignments and analyses of higher plant GLRs revealed that they group in three clades (Chiu *et al.*, 2002), with some authors proposing a fourth clade in monocotyledons (Chen *et al.*, 2016; De Bortoli *et al.*, 2016). Early land plants, such as mosses, ferns, and gymnosperms (e.g. ginkgo and pine tree with 9 and 40 GLRs, respectively), code for channels that exclusively cluster in clade 3, while clades 1 and 2 are only found in angiosperms (Price *et al.*, 2012; De Bortoli *et al.*, 2016) (Fig. 1). Functional data for plant GLRs is very limited, making it difficult to assess their overall or clade-specific role.

Early studies focused on pharmacology and point mutations to address structure–function relationships in iGluRs by patch-clamp (Traynelis *et al.*, 2010). More recent advances resolving iGluR structures by crystallography (Sobolevsky *et al.*, 2009; Chen *et al.*, 2014; Karakas and Furukawa, 2014; Yelshanskaya *et al.*, 2016) and cryo-electron microscopy (Zhao *et al.*, 2016; Twomey *et al.*, 2016; Lü *et al.*, 2017) positioned these proteins among the best-characterized ion channels at the molecular level (reviewed in Dawe *et al.*, 2015; Karakas *et al.*, 2015; Sobolevsky, 2015; Mayer, 2016; Zhu and Gouaux, 2017). The level of homology between plant GLRs and iGluRs has been accepted as a sufficient basis (Chiu *et al.*, 2002) to warrant inferences about their general structure derived from available data on iGluRs (Fig. 2). In the next paragraphs we will discuss the different GLR domains, including the N-terminal domain, the ligand-binding and pore domain, and the C-terminal domain (Fig. 2).

Structural and functional domains: iGluRs versus GLRs

The N-terminal signal peptide: to cleave or not to cleave?

Members of the iGluR family of ligand-gated ion channels generally possess an N-terminal transmembrane anchor

sequence that acts as a signal peptide allowing the nascent protein to enter the secretory pathway. Following the insertion of the protein into the endoplasmic reticulum (ER) membrane, the signal peptide is typically cleaved off and thus not part of the mature iGluR (He *et al.*, 2016). Based on *in silico* analyses of 18 different topology prediction programs (aramemnon.uni-koeln.de), 16 out of 20 *At*GLRs have a medium to high probability (predicted by ≥6 of 18 programs) of bearing an N-terminal transmembrane domain, which could function as a signal peptide (Table 1). Interestingly, among the *At*GLRs with a low probability (predicted by <6 programs), there are two *At*GLRs with predicted (*At*GLR2.5) or documented (*At*GLR3.5) localization outside the secretory pathway, namely in mitochondria and/or chloroplasts (Teardo *et al.*, 2015). Signal sequences mediating the targeting towards those organelles are typically soluble and eventually cleaved (Kim and Hwang, 2013), and therefore would not integrate into a membrane.

Whether these putative signal peptide sequences are cleaved is as yet not well studied, as very few conclusive experimental data are available to address this issue in plants. Experiments addressing this question either failed (Li *et al.*, 2006) or it was found that the first transmembrane domain alone was not necessarily sufficient for ER targeting (Davenport, 2002). Localization of *At*GLRs along the secretory pathway has only been shown for a few members, including *At*GLR1.4 (Tapken *et al.*, 2013), *At*GLR3.2, and *At*GLR3.3 (Vincill *et al.*, 2013, Wudick *et al.*, 2018), as well as *At*GLR3.4 (Meyerhoff *et al.*, 2005; Teardo *et al.*, 2011; Vincill *et al.*, 2012, 2013). For *At*GLR3.4 and an alternatively spliced *At*GLR3.5, localization in membranes of either chloroplast or mitochondria, respectively, was documented in addition to their plasma membrane localization (Teardo *et al.*, 2011, 2015), while *At*GLR2.1 is the first *At*GLR to be localized to the vacuolar membrane (Table 1) (Wudick *et al.*, 2018).

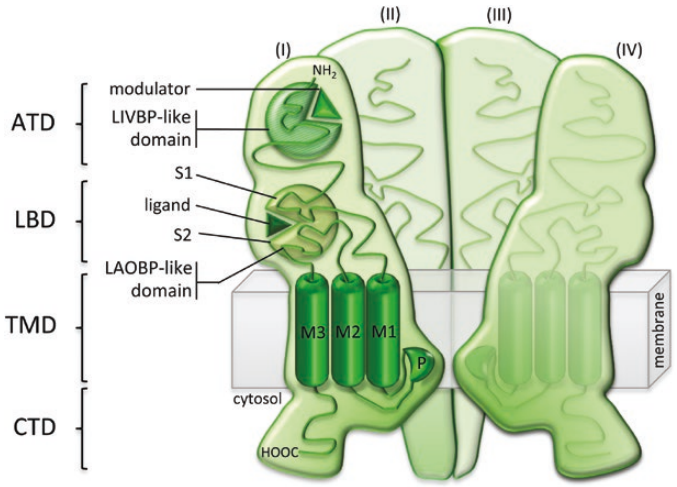

Fig. 2. Putative tetrameric membrane structure of *At*GLRs based on the structure of iGluRs. Schematic membrane structure of four *At*GLR subunits (I–IV). As exemplified in (I), each subunit consists of an amino-terminal domain (ATD) that forms a clamshell-shaped binding site for modulators with homology to bacterial leucine/isoleucine/valine-binding protein (LIVBP) domains. It is followed by the ligand-binding domain (LBD), which has a similar structure but the two lobes are composed of two segments (S1 and S2) from the soluble terminus and the external loop, respectively, and bear a lysine/arginine/ornithine-binding protein (LAOBP)-like domain with homology to the periplasmic binding protein-like II superfamily. The three membrane-spanning helices (M1–M3) of the transmembrane domains (TMDs) and a membrane re-entrant loop, forming the pore region (P) of the channel, are followed by the cytosolic carboxy-terminal domain (CTD). After Shepherd and Huganir (2007). Not drawn to scale. (This figure is available in colour at *JXB* online.)

The amino-terminal domain (ATD)

The ATD is located in the soluble N-terminus of glutamate receptors, and structures of several iGluR ATDs have been determined by crystallography, including members of the AMPA, kainate, and NMDA receptor family (reviewed in Dawe *et al.*, 2015; Karakas *et al.*, 2015; Sobolevsky, 2015; Mayer, 2016; Zhu and Gouaux, 2017). They were shown to form a clamshell-like binding pocket with homology to bacterial leucine/isoleucine/valine-binding protein (LIVBP) domains (Acher and Bertrand, 2005), which is absent in the bacterial iGluR0 (reviewed in Furukawa, 2012) (Fig. 2). The ATD is best studied in NMDA receptors where it is thought to allow regulation by allosteric modulators such as polyamines, Zn^{2+} ions, or specific agents such as ifenprodil (Paoletti *et al.*, 1997; Masuko *et al.*, 1999). So far no such regulation has been described for non-NMDA receptors, which might suggest that this layer of regulation is not present in AMPA and kainate receptors. Subunit assembly and multimerization of iGluRs in the ER as well as synaptic transmission were also shown to depend on the N-termini for members of the AMPA (Díaz-Alonso *et al.*, 2017), kainate (Sheng *et al.*, 2017), and NMDA receptor families (Traynelis *et al.*, 2010). Moreover, an alternatively spliced version of the ATD from the NMDA receptor GRIN1 was shown to affect the allosteric modification of the channel (Traynelis *et al.*, 1995). The ATD region additionally plays a role in iGluR gating (see below), as structure resolution by crystallography and cryo-electron microscopy revealed large movements of the region during pore opening. The opening is triggered by ligand binding, after which the channel spontaneously closes in a ligand-independent manner and transiently stays ligand insensitive. This mechanism, which is also referred to as desensitization, relies on specific molecular conformational changes involving the ATD domain (reviewed in Dawe *et al.*, 2015; Karakas *et al.*, 2015; Sobolevsky, 2015; Mayer, 2016; Zhu and Gouaux, 2017). Nevertheless, desensitization has not so far been unequivocally described for plant GLRs at the electrophysiological level, and there are no data indicating that the conformational changes in iGluRs are identical to those of plant GLRs.

Sequence comparisons revealed from the beginning an overall similarity between Arabidopsis GLRs and iGluRs (Lam *et al.*, 1998), and subsequent phylogenetic analyses indicated that both families diverged prior to the divergence of the iGluR classes (Chiu *et al.*, 1999). However, a later study refined the analyses by separately evaluating the relatedness of the first third (or N-terminal region) and the last two-thirds (or C-terminal regions) of *At*GLR proteins to iGluRs (Turano *et al.*, 2001). While supporting the conclusion that

the C-terminal regions were most closely related to iGluRs, the authors described that the N-terminal regions of *At*GLRs from clade 3 showed more homology to members of the subfamily C of G-protein-coupled receptors (GPCRs), which contain metabotropic glutamate receptors (mGluRs) and γ-aminobutyric $acid_B$ (GABA-B) receptors (Turano *et al.*, 2001). Accordingly, Gene Ontology annotation revealed a possible implication in GPCR-mediated ligand signaling for some *At*GLR3s (Roy and Mukherjee, 2016). Strikingly, this distinct ATD is absent in iGluRs but appears to be conserved in plant GLRs closely related to clade 3 *At*GLRs, including the moss *Physcomitrella patens*, the liverwort *Marchantia polymorpha*, and the gymnosperm *Ginkgo biloba* (Fig. 3), while being absent in clade 1 and 2 *At*GLRs. Interestingly, *At*GLR3.5 is the only member containing all amino acids of the domain's consensus motif. It has been reported that the conserved residues in this domain constitute a binding pocket for the glycine moiety (H_2N-RCH-COOH) of amino acids (Acher and Bertrand, 2005), and the binding of glutamate was shown for rat mGluR1 (Morikawa *et al.*, 2000). Based on these findings, it is tempting to speculate that members of the *At*GLR3 clade and other GLRs grouping with this clade are modulated or activated differently, not only when compared with iGluRs, but also when compared with members of clade 1 and 2 of *At*GLRs. Though lacking further characterization, several alternatively spliced versions of different *At*GLRs affecting the ATD are annotated (apps.araport.org/thalemine) and—if translated into functional proteins—are expected to change the modulation of the channels. An interaction of the N-terminus with auxiliary proteins similar to that described in iGluRs has not yet been reported for *At*GLRs.

Box 1

> *At*GLRs can have different target sequences and (additional) subcellular localizations
> They have a conserved iGluR/GLR ATD structure (clamshell domain), but are functionally different
> In NMDA receptors, the ATD is important for binding of allosteric modulators
> The ATD of *At*GLR3s bears a motif that is also found in metabotropic glutamate receptors (mGluRs) where it functions as a binding pocket for glycine moieties (i.e. amino acids)

The receptor or ligand-binding domain (LBD)

iGluRs are ligand-gated channels, which upon binding of the agonist open their pore. Decades of point mutation analyses revealed two protein segments, S1 and S2, that are composed of two highly conserved 10 amino acid motifs, which are directly involved in ligand binding and flank the

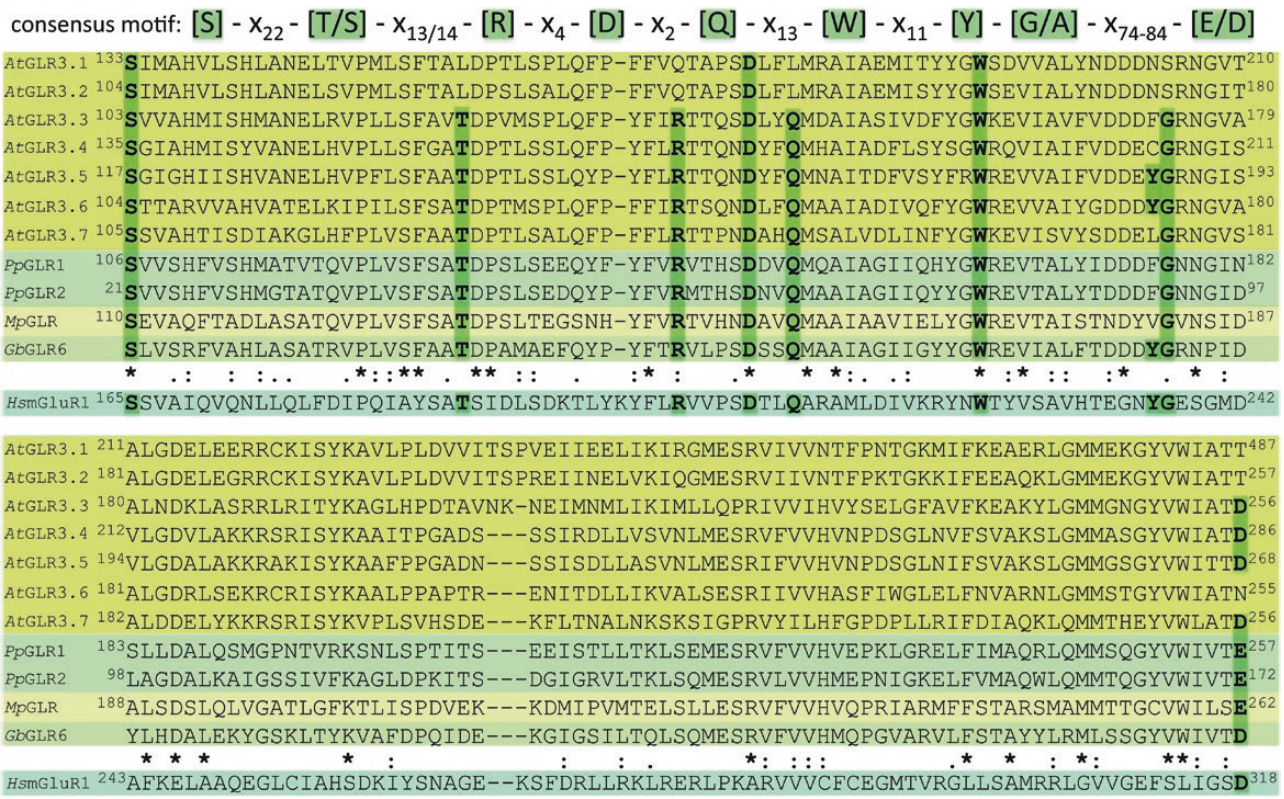

Fig. 3. Predicted amino-terminal domain (ATD) in the N-terminus of clade 3-like GLRs and metabotropic glutamate receptors (mGluRs). Sequence alignment of the N-terminal domains of clade 3-like GLRs from *Arabidopsis thaliana* (*At*), *Physcomitrella patens* (*Pp*), *Marchantia polymorpha* (*Mp*), and *Ginkgo biloba* (*Gb*), as well as the human metabotropic glutamate receptor 1 (*Hsm*GluR1). The residues of the consensus sequence (on the top and bold letters with green highlight) are predicted to form a binding pocket for glycine moieties. Conservation of plant ATDs: *fully conserved residue; : conservation between groups of strongly similar properties; . conservation between groups of weakly similar properties.

membrane pore-forming domain of the channel (Traynelis *et al.*, 2010) (Fig. 2). The 3D structure of the S1 and S2 stretches is buried inside a receptor domain, which, like the ATD, folds into a clamshell-like structure (reviewed in Dawe *et al.*, 2015; Karakas *et al.*, 2015; Sobolevsky, 2015; Mayer, 2016; Zhu and Gouaux, 2017). This structure is homologous to lysine/arginine/ornithine-binding protein (LAOBP)-like domains from members of the periplasmic-binding protein-like II superfamily in bacteria (Acher and Bertrand, 2005). When the ligand binds, the clamshell closes, which induces a larger trans-conformational rearrangement of the tetrameric channel, and leads to the opening of the pore (Mayer, 2016; Zhu *et al.*, 2016; Twomey and Sobolevsky, 2018). Animal iGluRs show strong ligand specificity. Depending on their subunit composition, NMDA and delta receptors bind either to glutamate/aspartate or to glycine/D-serine, while AMPA and kainate receptors exclusively bind to glutamate/aspartate (Traynelis *et al.*, 2010). The binding displays low affinity, which occurs typically at ~0.1–3 μM for endogenous ligands (Traynelis *et al.*, 2010), and its specificity and affinity are determined by the structure formed by S1 and S2 inside the clamshell. The S1 and S2 domains are highly conserved among iGluRs, showing the highest conservation within each of the iGluR clades.

Strikingly, a similar sequence conservation is missing in S1- and S2-like domains of *At*GLRs (De Bortoli *et al.*, 2016). The gating of *At*GLRs is still not well characterized (Forde and Roberts, 2014) and was initially assessed by employing the pharmacology from iGluRs. The first indication of possible GLR activity in plants was detected as an effect on hypocotyl growth in relation to light upon application of the iGluR antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX; Lam *et al.*, 1998). However, while the AMPA receptor-specific antagonist DNQX is typically used at concentrations of 10 μM when studying iGluRs (affinity <1 μM; Traynelis *et al.*, 2010), much higher concentrations of ~0.1–0.4 mM were initially used in plants. Accordingly, later studies in plants showed a lack of specificity for antagonists of the animal NMDA or AMPA receptor class, with or without associated agonists. Examples include studying the effects of cyanquixaline (6-cyano-7-nitroquinoxaline-2,3-dione) (CNQX), DNQX, and 2-amino-5-phosphonovalerate (AP5) on tobacco pollen tubes and protoplasts (Michard *et al.*, 2011), of DNQX on whole plants (Dubos *et al.*, 2003), of 500 μM CNQX, DNQX, or 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) on leaves (Meyerhoff *et al.*, 2005), of 100 μM DNQX on vesicles from the inner chloroplast membrane of spinach containing *At*GLR clade 3-like receptors (Teardo *et al.*, 2010), or of AP5 and DNQX on leaves from *glr3.3* mutant plants treated with the potential ligand glutathione (Li *et al.*, 2013). Moreover, the palette of putative endogenous *At*GLR agonists is not yet well defined. First proof of a plant GLR activity was shown by using the cycad-derived agonist β -*N*-methylamino-L-alanine (BMAA) to block light-induced hypocotyl shortening and cotyledon expansion in Arabidopsis (Brenner *et al.*, 2000). Subsequent studies revealed that plant GLRs seem to respond to a wide range of amino acids, expanding beyond the name-giving glutamate and including non-conventional amino acids, such as D-serine, and the tripeptide glutathione (Michard *et al.* 2011; Weiland *et al.*, 2016). Pharmacological analyses of *At*GLRs are consistent with the idea of them being different from non-NMDA receptors in general and divergent within plant GLR clades in particular.

In animals it was shown that in addition to glutamate, many other amino acids and naturally occurring molecules are able to activate AMPA and kainate receptors (Traynelis *et al.*, 2010), making it difficult to pinpoint specific mechanisms related to each agonist. So far only *At*GLR1.4 and 3.4 have been characterized as ligand-gated channels in heterologous systems [for a thorough review on *At*GLR (ant)agonists, see Weiland *et al.*, 2016]. *At*GLR1.4 can be activated by methionine, tryptophan, phenylalanine, leucine, tyrosine, asparagine, and threonine, but, when studied in seedlings, only methionine was found to have a depolarizing effect on the membrane potential, which was absent in a *glr1.4* mutant (Tapken *et al.*, 2013). It is antagonized by eight natural amino acids, arginine being the most efficient, as well as by the synthetic antagonists DNQX, Philanthoxin, and MK-801, although at high concentrations (≥100 μM; Tapken *et al.*, 2013). By introducing point mutations, the authors proved the involvement of the LBD in ligand binding to *At*GLR1.4 (Tapken *et al.*, 2013). Upon heterologous expression in HEK cells, *At*GLR3.4 could be activated by asparagine, L-serine, and glycine (Stephens *et al.*, 2008). Of note, the ligand concentrations used in these experiments ranged widely between micromolar (*At*GLR1.4) and millimolar (*At*GLR3.4) concentrations. Intriguingly, and in contrast to their animal homologs, some GLRs also seem to be active without the presence of a ligand (Michard *et al.*, 2011; Ortiz-Ramírez *et al.*, 2017, Wudick *et al.*, 2018). In tobacco pollen tube protoplasts, the antagonist CNQX inhibited currents, which were recorded in the absence of any ligand in the bath solution (Michard *et al.*, 2011). Even more strikingly, *At*GLR3.2 and 3.3, as well as *Pp*GLR1 from the moss *P. patens*, induced ionic currents without the presence of any ligand when expressed in COS-7 cells (Ortiz-Ramírez *et al.*, 2017, Wudick *et al.*, 2018). In the case of *Pp*GLR1, the currents were inhibited by the iGluR antagonists AP5 and CNQX, suggesting that the channel was open in a basal state configuration, without any apparent ligand (Ortiz-Ramírez *et al.*, 2017). Altogether, the data suggests that gating of plant GLRs is different from that observed in animal iGluRs. Further functional characterization and structural analyses are necessary to understand fully the gating, or apparent lack thereof, in plant GLRs.

The pore: driving ionic selectivity

The ionic pore is formed at the interface of the four subunits of the functional channel (Fig. 2). On the protein sequence level, the pore region is composed of ~20 residues located between the first and second transmembrane domains, forming a membrane re-entrant loop (Traynelis *et al.*, 2010). While the prokaryotic iGluR0 is a highly potassium-selective channel and shows the typical 'GYGD' selectivity filter motif (Chen *et al.*, 1999), which is also found in rotifer *Av*GluR1 (Janovjak *et al.*, 2011), related animal iGluRs lost that motif and they are non-selective cation channels (Traynelis *et al.*, 2010). Some

iGluRs, especially from the NMDA family, were shown to be Ca^{2+} permeable (Traynelis *et al.*, 2010). The pore from an AMPA receptor in an open state has recently been resolved by cryo-electron microscopy imaging (Twomey and Sobolevsky, 2018). Of special note, RNA editing mechanisms affecting residues from the pore (and the extracellular loop) region of iGluRs have been described to modulate this Ca^{2+} permeability, and channel properties in general (Barbon and Barlati, 2011). The Q/R editing site in the pore region of AMPA and kainate receptors represents some of the best-characterized residues with implication in several diseases (Barbon and Barlati, 2011). A similar kind of editing has not so far been documented for *At*GLRs. Unfortunately, the poor conservation of the pore region between animal and plant glutamate receptors makes it impossible to derive information on GLR selectivity (De Bortoli *et al.*, 2016). In addition, the pore sequence, and particularly the residues dictating the Ca^{2+} permeability in NMDA channels, are poorly conserved in *At*GLRs, suggesting an overall different selectivity for GLRs, and possibly large selectivity differences between plant GLRs (De Bortoli *et al.*, 2016).

With a few exceptions, the selectivity of *At*GLRs is unknown. Ca^{2+} imaging and studies on the membrane electric polarization of leaf cells in response to amino acids in wild-type and *glr3.3/3.4* knock-down plants suggested that those channels are Ca^{2+} permeable and could be blocked by the broad cation channel inhibitor gadolinium (Gd^{3+}, Kim *et al.*, 2001). In pollen tubes, D-serine increased Ca^{2+} influxes as measured by using external ion-specific electrodes, as well as cytosolic Ca^{2+} concentrations monitored with the yellow cameleon Ca^{2+} probe YC3.6 (Michard *et al.*, 2011). The first molecular data were obtained by swapping the pore of 17 *At*GLRs with those of AMPA and kainate receptors, followed by the characterization of the HEK cell-expressed chimera by the patch-clamp technique (Tapken and Hollmann, 2008). Two chimeras containing the pores of *At*GLR1.1 and 1.4 were functional, both displaying a non-selective cationic conductance. Furthermore, expression in HEK cells of *At*GLR3.4 was found to generate a cationic current, partly carried by Ca^{2+} (Vincill *et al.*, 2012). A similar result was described for *At*GLR1.4 upon expression in *Xenopus* oocytes (Tapken *et al.*, 2013). Recent characterization of the moss channel *Pp*GLR1 and of *At*GLR3.2 and 3.3 showed that these channels are permeable to cations, including Ca^{2+}, which could be partially inhibited by Gd^{3+} (Ortiz-Ramírez *et al.*, 2017; Wudick *et al.*, 2018). In summary, essential questions such as *At*GLR permeability to Ca^{2+}, relative selectivity for different ions, anion permeability, and the existence of a mechanism similar to the magnesium block of NMDA receptors (Mayer, 2017) to avoid a toxic Ca^{2+} influx into the cytosol remain unanswered and need to be addressed in the future.

The 'gate' region of the channel

In a simplistic way, structural changes induced by agonist binding to the LBD are transmitted to the 'gate' region of the protein, which is occluding the pore in a non-ligand-bound state, leading to the opening of the pore of the channel (Twomey and Sobolevsky, 2018). Wider conformational changes occur in response to receptor–gate interactions, including large rearrangements between the subunits of the channel tetramer, and the twist of the NTD domain (reviewed in Dawe *et al.*, 2015; Karakas *et al.*, 2015; Sobolevsky, 2015; Mayer, 2016; Zhu and Gouaux, 2017). In iGluRs, the 'gate' is highly conserved and represented by a 'SYTANLAA' amino acid motif (Traynelis *et al.*, 2010) (Fig. 4). Targeted mutations in the 'gate' motif of GRIA2 revealed that the second alanine is responsible for 'gate' opening (Moore *et al.*, 2013).

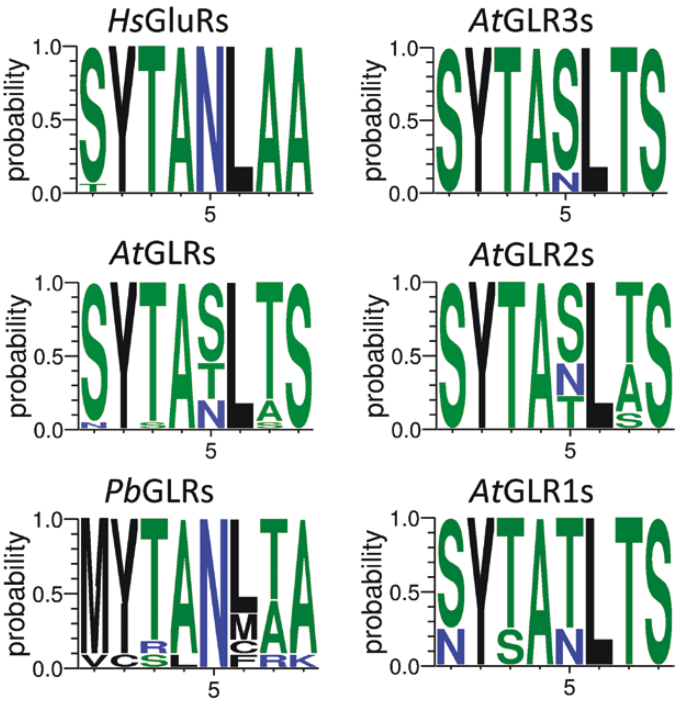

Fig. 4. Graphical representation of the sequence conservation for the GLR 'gate' motif in different species. Representation of aligned sequences (numbers given) from the 'gate' region of all human iGluRs (*Hs*GluRs, 18), *Arabidopsis thaliana* (*At*GLRs, 20), and *Pleurobrachia bachei* (*Pb*GLRs, 11) (left column), and a clade-specific representation of *At*GLRs (right column). The 'gate' motif of all nine ginkgo *Gb*GLRs, the two moss *Pp*GLRs, and the algal *Mp*GLR represented in Fig. 1 are identical to the *At*GLR3 consensus motif, except for a perfectly conserved serine (S) at position 5. *At*GLR3.7 is the only *At*GLR3 with an asparagine (N) in this position. Note that two *Pb*GLRs did not display any 'gate' motif and were not considered here. Logos were created with WebLogo3.5 (Crooks *et al.*, 2004).

Sequence comparisons between animal iGluRs and plant GLRs reveal that the 'gate' motif is not completely conserved (Fig. 4). In *At*GLRs, only the tyrosine (T), alanine (A), and leucine (L) in position 3, 4, and 6, respectively, are fully conserved, and most variability is observed in position 5 (Fig. 4). Interestingly, within Arabidopsis, *At*GLR3s show the most conserved 'gate' motif, that—except for *At*GLR3.7—follows the 'SYTASLTS' sequence, which is also conserved in the two moss and all nine ginkgo GLRs (Fig. 4). While *At*GLR2s show additional variation in position 7, *At*GLR1s show by far the most divergent 'gate' motif, with further variations in positions 1 and 3 (Fig. 4). Interestingly, in iGluRs, an A to T switch in position 8 of the motif, the so-called *Lurcher* mutation, results in a constitutively open channel (Zuo *et al.*, 1997; Klein and Howe, 2004). Since several plant GLRs display a serine in position 8, which like threonine is a polar, hydrophilic amino

acid, it is tempting to speculate that at least some of them could also display channel activity in the absence of any agonists. Indeed, the moss *Pp*GLR1 as well as *At*GLR3.2 and 3.3 were recently shown to be active without any ligand (Ortiz-Ramírez *et al.*, 2017; Wudick *et al.*, 2018), indicating that ligand-dependent channel activation might not be necessary in plant GLRs or may have been lost in other species. An extreme example of the latter can be found for *Pleurobrachia bachei*, which displays very poor conservation of the 'gate' motif (Fig. 4).

Box 2

> Unlike their homologous domains in iGluRs, S1/S2 segments are poorly conserved in GLRs
> There is an apparent broad ligand spectrum for *At*GLRs
> The pore region is poorly conserved in plant GLRs
> The divergent 'gate' motif might reflect that some GLRs (i.e. *Pp*GLR1, *At*GLR3.2, and *At*GLR3.3) might function without ligand-induced activation

From the 'gate' to the cytosolic C-terminus: a role in gating and desensitization

A region including the S2 lobe of the LBD and preceding the last transmembrane domain of AMPA receptors was shown to undergo alternative splicing events, yielding either 'FLIP' or 'FLOP' variants of the channel. Though the physiological role of this event is still not totally understood, the two splicing variants display different kinetic properties and show differential interaction with auxiliary proteins binding in that region (Greger *et al.*, 2017). An impact on the early trafficking of the channel was also reported (Coleman *et al.*, 2006). With the exception of the N-terminal region of *At*GLR3.5 (see above), alternative splicing events have not been studied for plant GLRs, but the occurrence of several splicing variants covering the respective regions in *At*GLRs (apps.araport.org/thalemine) might be indicative of a similar way to modify the channel activity/specificity. Interestingly, in the case of *At*GLR2.4, the most abundant (and hence the often only annotated) splicing variant is completely lacking the 'gate' motif.

While present in both iGluRs and GLRs, the last transmembrane domain is absent in the prokaryotic iGluR0 (Chen *et al.*, 1999). In addition to serving as an 'anchor' for the C-terminal domain that is essential for iGluR post-translational regulation (see below), it is also involved in conformational changes of the channel during gating, as revealed by point mutation analyses in iGluRs. More specifically, tryptophan-scanning mutagenesis of the last transmembrane domain revealed its impact on gating (for both NMDA and AMPA receptors) and desensitization (for AMPA receptors) (Amin *et al.*, 2017). Importantly, this desensitization is essential for iGluR function in general, and their clade-specific function in particular (Traynelis *et al.*, 2010; Popescu, 2012). In leaf cells, an amino acid-specific desensitization-like phenomenon has been reported, which was affected in *glr3.3* and *glr3.4* knock-down plants, suggesting that a desensitization-like mechanism might also exist in plants (Stephens *et al.*, 2008). Nevertheless, neither *At*GLR1.4 nor 3.4 showed desensitization kinetics when heterologously expressed (Vincill *et al.*, 2012; Tapken *et al.*, 2013).

The cytosolic C-terminal domain

The intracellular C-terminus of iGluRs is the most diverse part of the receptors, in terms of both amino acid sequence and length (Traynelis *et al.*, 2010). Despite the apparent lack of functional domains, distinct motifs such as ER retention sites can be found (Traynelis *et al.*, 2010). Additionally, some AMPA receptors also harbor a C-terminal consensus motif, which allows interaction with proteins bearing a Psd-95/DlgA/ZO1 (PDZ)-domain, such as GRIP, ABP/GRIP2, PICK1, and others, which are involved in trafficking and recycling of the receptor (Collingridge *et al.*, 2004; Shepherd and Huganir, 2007; Traynelis *et al.*, 2010). Interestingly, iGluR interaction/modification through PDZ domains is apparently not well conserved or only occurred in higher organisms, since none of the *Drosophila* iGluRs from the neuromuscular junction have such a domain (Kim *et al.*, 2012). Accordingly, so far neither homologs nor proteins with PDZ domains were identified in Arabidopsis. The presence of 14-3-3 protein-binding sites in the C-terminus of several iGluRs has also been documented. While interaction with 14-3-3 proteins and the kainate receptor GRIK2 slowed down the receptor's decay kinetics (Sun *et al.*, 2013), interaction with members of the NMDA receptor family impacted the trafficking and isoform-specific interaction (Chung *et al.*, 2015) or led to increased surface expression of the channels (Chen and Roche, 2009). Accordingly, deletion of the C-terminus did not abolish the overall function but rather affected the regulation of different iGluRs (Traynelis *et al.*, 2010).

Similar to their mammalian counterparts, *At*GLRs show highly variable C-terminal sequences and lengths (Fig. 5). While *At*GLR1s display the shortest C-termini (18 amino acids), the lengths of the C-termini are most divergent in *At*GLR2s (between 41 and 113 amino acids; Fig. 5). Multiple putative ER retention/retrieval motifs with documented function in plants (i.e. KKxx, KxKxx, RR, RxR, RxxR, and ΦxxΦ[K/R/D/E]) (Boulaflous *et al.*, 2009; Gao *et al.*, 2014) are present in all *At*GLR C-termini (Fig. 5). Moreover, 14-3-3 protein mode I (Rxx[S/T]xP) or mode II (Rxxx[S/T]xP) binding sites can be found in the C-termini of six *At*GLRs from all three clades (Table 2). For three members (*At*GLR1.4, 2.9, and 3.7), an interaction with 14-3-3 proteins was shown experimentally (Chang *et al.*, 2009; Shin *et al.*, 2011) (Table 2). Since binding of 14-3-3 proteins relies on phosphorylation of the serine/threonine residue in the center of the binding motif, phosphorylation events are also likely to occur in the GLR C-terminus. An interaction of the C-termini with other auxiliary proteins is possible but not yet documented.

Box 3

> *At*GLR C-termini are most variable in terms of sequence and length
> They contain putative ER retention motifs and 14-3-3 protein-binding motifs
> They lack regulatory PDZ domains that can be found in iGluRs

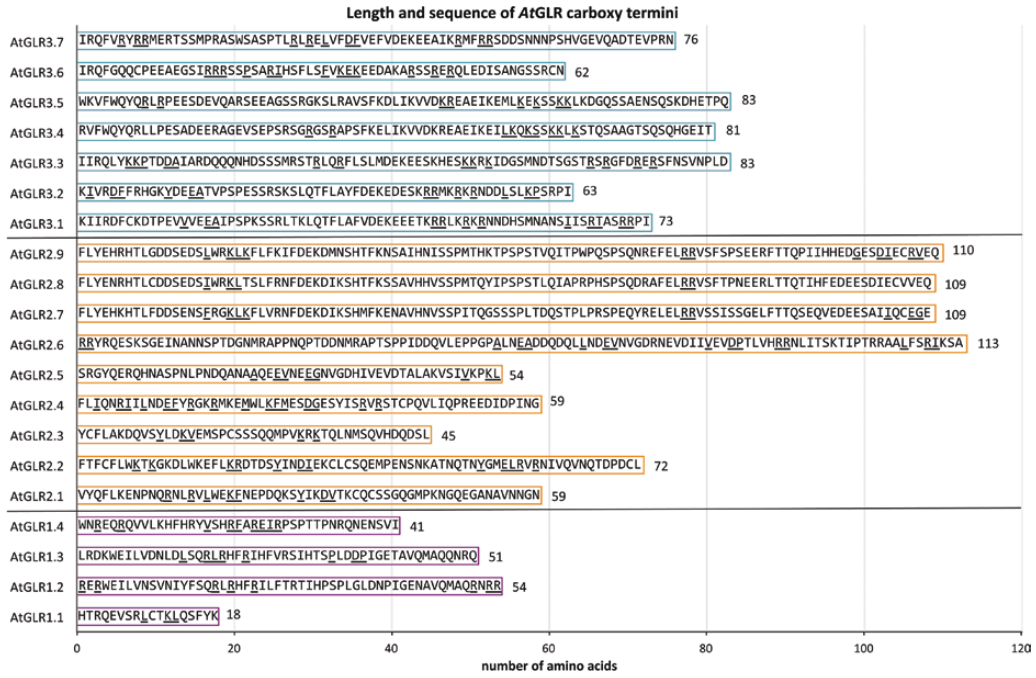

Fig. 5. Graphical representation of the length and sequence of *At*GLR C-termini. The graph depicts the lengths and sequences of all 20 *At*GLR C-termini. While clade 1 termini (magenta) are overall the shortest, the length of clade 2 C-termini (orange) is most divergent. The amino acid number is given for each peptide. Lengths of clade 3 C-termini are contoured in blue. Putative ER retention motifs of the KKxx, KxKxx, RR, RxR, RxxR, or Φxx[K/R/D/E]Φ type are underlined. The lengths were determined with the help of multiple topology prediction programs at aramemnon.uni-koeln.de.

Table 2. *At*GLRs with a putative C-terminal 14-3-3 protein-binding motif

Gene	Position	Peptide	Mode	Interacting 14-3-3 protein
*At*GLR1.3	840	FV**R**SIH[**T**]S**P**LD	II	–
*At*GLR1.4	850	EI**R**PSP[**T**]T**P**NR	II	At4g05685, *At*GRF1[a]
*At*GLR2.6	832	DNM**R**AP[**T**]S**P**PI	I	–
*At*GLR2.9	921	SEE**R**FT[**T**]Q**P**II	I	At1g78300, *At*GRF2[b]
*At*GLR3.6	861	GSI**RR**R[**S**]S**P**SA	I or II	–
*At*GLR3.7	860	RME**R**TS[**S**]M**P**RA	I	At1g78300, *At*GRF2[b]

The table lists *At*GLRs with predicted C-terminal 14-3-3 protein-binding motifs and their position. Conserved residues are in bold, and putatively phosphorylated residues in brackets. The motif modes (I Rxx[S/T]xP; or II, Rxxx[S/T]xP) and experimental evidence for interaction are given. [a] Shin *et al.* (2011); [b] Chang *et al.* (2009).

From structure to function: implications of plant GLRs

GLRs play a role in Ca^{2+} signaling

Although the pivotal role of Ca^{2+} signaling during plant development and (a)biotic stress responses has been acknowledged and documented for years, the discovery and characterization of the molecular nature of possible Ca^{2+} transport systems spanned decades and is still ongoing. GLRs were one of the first molecular candidates that were shown to transport Ca^{2+} (Michard *et al.*, 2011;Vincill *et al.*, 2012), and since have been linked to Ca^{2+} influx into leaf cells and during plant immune responses (reviewed by Forde and Roberts, 2014), for example. With documented plasma membrane localizations, and considering the fact that mutations in *GLR* genes affect chemotaxis and immune response pathways, it is plausible that GLRs are involved in plasma membrane-elicited pathways that are triggered by ligands from environmental cues. Following the pathogen–plant interaction studies involving GLR mutants (Kang *et al.*, 2006; Li *et al.*, 2013; Manzoor *et al.*, 2013), subsequent studies tried to understand whether GLRs could recognize microbe-associated molecular patterns (MAMPs), which often trigger the innate immune response pathways. Despite being addressed by an indirect approach when using pharmacology to block GLRs, it was shown that *At*GLRs partly participate in the MAMP recognition and consequent apoplastic Ca^{2+} influxes that are necessary for the activation of downstream signaling events [mitogen-activated protein kinase (MAPK)] related to plant defense (Kwaaitaal *et al.*, 2011). It was further shown that GLRs regulate pollen tube growth by controlling Ca^{2+} fluxes at the pollen tube tip (Michard *et al.*, 2011;Wudick *et al.*, 2018) and participate in sperm cell guidance (Ortiz-Ramírez *et al.*, 2017). More specifically, it was shown that the amino acid D-serine acts as an agonist for pollen GLRs, which are believed to be involved in pollen tube growth control (Michard *et al.*, 2011). Moreover, *At*GLRs are involved in generating the Ca^{2+} wave in response to aphid feeding in leaves (Vincent *et al.*, 2017).

The main physiological role of iGluRs is voltage control. What about GLRs?

All documented physiological implications of *At*GLRs so far are linked to their capability to permeate Ca^{2+}. Strikingly, in animals, this role is mostly attributed to NMDA receptors, while non-NMDA receptors show a much more diversified ion selectivity (Traynelis *et al.*, 2010).

As exemplified before, animal iGluRs have been extensively studied in neurons, highlighting their role in regulating membrane potential and fast excitatory synaptic transmission (Traynelis *et al*., 2010). In synapses, upon agonist binding, iGluR transmembrane domains change their conformation, opening a pore on the membrane that allows the influx of K^+, Na^+, and/or Ca^{2+}, depending on the selectivity of the channel (Willard and Koochekpour, 2013). The influx of cations, consequently, depolarizes the membrane, elevating the membrane potential to values closer to the depolarized voltage threshold, which in turn activates voltage-gated channels in the vicinity, eventually generating an action potential (Traynelis *et al*., 2010). Studies in mouse giant synapses revealed that knocking down the only iGluR expressed in those cells almost totally abolished excitatory post-synaptic currents and delayed the onset of action potential generation (Seol and Kuner, 2015). This work exemplarily showed that iGluRs are the first responders to neurotransmitter-conveyed signals by bringing the membrane potential close to the threshold, thus priming the cell to fire an action potential. Despite being crucial for cell physiology, membrane potential fluctuations have been underappreciated for a very long time (Blackiston *et al*., 2009; Gallé *et al*., 2015).

Interestingly, observations in plants also point to the importance of membrane potential and changes thereof for plant physiology. For instance, mesophyll cells were shown to change their membrane potential after dark and light transitions (Shabala and Newman, 1999). It was further shown in guard cells that a stimulus-induced membrane depolarization triggered an activation of anion channels, which further depolarized the membrane, resulting in an increase of the osmotic potential and subsequent closure of the stomata (Hedrich, 2012). It is interesting to note that some of the stimuli triggered a Ca^{2+}-mediated response and that changes in the membrane voltage played an essential role in this process, during both the signaling (through voltage-gated channels) and the response (increase of water potential) (Hedrich, 2012).

Even though plants do not have such specialized cells as neurons, they do have excitable cells capable of generating and propagating electrical signals (Gallé *et al*., 2015; Hedrich *et al*., 2016). In fact, bio-electric phenomena in plants were widely characterized more than a century ago (i.e. Bose, 1913). The closest structure to axons in plants is the phloem, a symplastic tissue, displaying a low-resistance path for electric signaling. During their development, the phloem cells undergo partial programmed cell death resulting in the degeneration of the central vacuole, the nucleus, and the common plastids, thereby creating a low resistance cytoplasm dominated by an electrolyte of ~100 mM K^+ and thus more favorable to conduct electrical signals (Gallé *et al*., 2015). It was recently shown that *At*GLRs are crucial to evoke and propagate an electrical signal along the phloem in response to herbivory and that mutations in *AtGLR3.1*, *3.2*, *3.3*, and *3.6* caused reduced duration of surface potential changes in Arabidopsis leaves after caterpillar-induced wounding (Mousavi *et al*., 2013). Strikingly, the propagation of the electrical signal to neighboring leaves was dependent on *AtGLR3.3* and *3.6*, showing the implication of those genes in conveying the electrical signal over a long distance (Mousavi *et al*., 2013). Moreover, *At*GLR3.5 was shown to block the spatial distribution of those long-distance electrical signals, preventing the signal from being fully systemic. Conversely, in *glr3.5* mutants, wound-induced action potentials transversed the entire plant, propagating the electrical signal to non-neighboring leaves (Salvador-Recatalà, 2016). Overall, those results showed that *At*GLRs can modulate the shape of the electrical signal and its intensity, and further control its propagation to defined organs, thus providing evidence for a tight regulation of *At*GLR-mediated electrical signals that may be as complex as in neurons.

Beyond the neurons: conclusions and outlook for a complex family

In addition to playing a pivotal role in the central nervous system, iGluRs are also expressed in many peripheral, non-neuronal cells. For instance, in bones, iGluRs were shown to influence the dynamic remodeling of this tissue (Hinoi *et al*., 2004; Xie *et al*., 2016). In the pancreas, iGluRs are important for the ion signaling in the islet β-cells that control insulin release (Hinoi *et al*., 2004), while in kidneys iGluRs stimulate vasodilation in the glomerulus, which may have an impact in water/salt balance, potentially regulating blood pressure (Hogan-Cann and Anderson, 2016). Interestingly, it was shown that peripheral iGluRs act in heteromers different from those in the central nervous system, and that their kinetics, especially of desensitization, appear to be slower (Traynelis *et al*., 2010). Consequentially, understanding the physiological role of iGluRs outside the nervous system becomes more and more important. It is noteworthy that the characterization of these peripheral iGluRs faces limitations and problems similar to the study of plant GLRs. (i) Peripheral iGluRs seem to be involved in Ca^{2+} signaling, but the molecular mechanism are not understood, though it has been proposed that they could modulate the basal Ca^{2+} level in the cell. (ii) The amino acid concentration in the periphery of cells that express those iGluRs is much higher than their half-activation concentration observed in the brain, particularly in red blood cells or lymphocytes that evolve in an environment of constantly high amino acid concentrations (Zhou *et al*., 2013). So how does the gating of those channels work? (iii) Complex subunit arrangements and multimer formation might occur and alter channel properties.

In this review, we discussed that GLRs and iGluRs, although sharing a common structure, display major sequence divergences, especially in key domains such as the receptor, the pore, and the 'gate'. Recent electrophysiological characterization of *Pp*GLR1, *At*GLR3.2, and *At*GLR3.3 (Ortiz-Ramírez *et al*., 2017; Wudick *et al*., 2018) showed their ligand-independent activity, providing evidence for functional differences between iGluRs and at least these specific GLRs. In addition to the GLR diversity (20 members in Arabidopsis, four clades in higher plants), *At*GLRs can apparently undergo various alternative splicing processes at the mRNA level, and have the ability to form clade-overarching functional heteromers (Price *et al*., 2013;Vincill *et al*., 2013).

The aforementioned differences between iGluRs and plant GLRs make it obvious that more functional data are required to understand this complex channel family profoundly on the cellular level. Generally, functional comparisons between GLRs and iGluRs should be done very carefully, since both families seem to have at least as many things in common as things that separate them.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Sequences used to generate the phylogram, gate motif logos, and ATD regions.

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