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Review

Signalling via glutamate and GLRs in *Arabidopsis thaliana*

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Abstract. The genome of *Arabidopsis thaliana* (L. Heynh.) contains 20 coding sequences for homologues of animal ionotropic glutamate receptors. These glutamate receptor-like receptors act as sensors and mediators of a multitude of exogenous as well as endogenous signals and are found in all analysed plant species. Their molecular structure clearly indicates a function as integral membrane proteins with a ligand-gated ion channel activity. Altered gene expressions and the occurrence of mRNA splice variants confer a high flexibility on the gene as well as on the RNA level. An individual glutamate receptor of *A. thaliana* is able to bind two different ligands (most probable amino acids and their derivatives), whereas a functional receptor complex is likely to consist of four single proteins. These features enable an immense number of sensitivities against various local and temporal stimuli. This review encompasses the last 15 years of research concerning glutamate signalling and glutamate receptors in plants. It is aimed at summarising their major characteristics and involvements to obtain a broader and farer reaching perspective of these fundamental components of plant signal transduction.

Additional keywords: amino acid, calcium, electrophysiology, GLR, ion channel, signaling.

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Introduction

Since the discovery of 20 genes/proteins in *Arabidopsis thaliana* (L. Heynh.) as homologues of ionotropic glutamate receptors (iGluRs) (Lam *et al*. 1998), numerous studies have been published. These elucidate their phylogenetic emergence, physiological functions, as well as multiple roles of these receptors in cellular and electric long-distance signalling evoked by glutamate (Price *et al*. 2012; Forde *et al*. 2013; Mousavi *et al*. 2013; Price and Okumoto 2013; Vincill *et al*. 2013). Products of these genes were named glutamate-receptor-like receptors (GLRs) due to a high similarity to their animal counterparts in respect to their nucleotide and amino acid sequence (Lacombe 2001). This similarity ranges from 16 to 63% within the ligand binding domains S1 and S2 and the transmembrane domains M1 to M4 when compared with (*2R*)-*2*-(*methylamino*) *butanedioic acid* (NMDA) receptors in animals. Whereas a part of the pore-forming M3 domain features the highest level of identity (Lam *et al*. 1998). Glutamate receptors of *A. thaliana* (AtGLRs) act as sensors and mediators for a multitude of exogenous and endogenous signals in plants. The structure of these proteins indicates a function as integral membrane proteins with a ligand-gated ion channel activity. Plant GLRs mediate early Ca^{2+} fluxes across membranes, after their activation by respective ligands, and control a plethora of physiological and developmental events downstream of the calcium signalling. Thorough understanding of glutamate signalling and GLRs in plants is of eminent importance to explain several plant functions encompassing long-distance signalling, plant development, plant nutrition, stress adaptation, photosynthesis, carbon metabolism, plant stress adaptation as well as symbiotic and immune defence reactions.

Analyses on the expression levels point to a highly dynamic system in which GLR gene expression responds to external and internal stimuli to the plant. The occurrence of mRNA splice variants of some AtGLR genes extends their flexibility from the gene to the RNA level. Their molecular structure allows a single GLR to bind two different ligands while a functional receptor complex in *Arabidopsis* is likely to consist of four individual GLRs (Roy *et al*. 2008). All these features together enable a virtually endless number of local and temporal sensitivities to various stimuli within plant organisms.

The 20 GLRs of *A. thaliana* can be grouped into three clades based on DNA sequence similarities (Chiu *et al*. 2002). AtGLR clade I and II are sister clades and it is assumed that clade II originates from clade I and came into existence by gene duplication events (Chiu *et al*. 2002). Indeed, it seems that many of the GLRs are actually duplicated genes as they often exist in tandems on single chromosomes (Chiu *et al*. 2002; Singh *et al*. 2014). Gene duplication could be the driving force for the evolution and expansion of GLRs in general since these proteins

exist abundantly in most plant species. The analysis of AtGLR gene expressions indicates that originally all GLRs were expressed in roots, leaves and reproductive organs where they fulfilled essential tasks (Kim *et al.* 2001; Chiu *et al.* 2002; Meyerhoff *et al.* 2005). Today, the AtGLR genes of clade II are no longer expressed throughout the whole plant and it is possible that their functions are substituted by members of the other clades (Chiu *et al.* 2002; Pina *et al.* 2005). So far, it is not known if clade II is specialised on distinct functions. There are few publications concerning this clade, and no distinct phenotypes could be observed until now.

Molecular structure

AtGLRs are made of between 800 and 960 amino acids and each receptor has a molecular weight of ~100 kDa. They contain six conserved domains that are found also in iGluRs of animals (Lam *et al.* 1998). These domains can be functionally divided into two extracellular ligand-binding sites (S1, S2) for ligand binding and four transmembrane domains (M1–M4) that enable the passage of ions (Fig. 1). In animals, the binding of a molecule at the ligand-binding domains S1 and S2 leads to conformational modifications that affect the whole receptor and alters the position of secondary structures near the pore region. These changes are essential for the passage of ions across plasma membranes (Sobolevsky *et al.* 2009). The *N*-terminal region contains a further ligand binding site, enabling AtGLRs to bind another agent that could act as either an agonist/antagonist) or as a modulator for the receptor functionality. The whole *N*-terminal domain is exposed to the exterior site of the membrane in animals and plants. Therefore, it is possible that these receptors function in a similar way in both animal and plant kingdoms.

It is believed that a fully operative plant GLR receptor is made of at least four subunits comparable to iGluRs in animals (Dubos *et al.* 2003). Members of the NMDA receptor family are seen as the closest relatives to AtGLRs. NMDA receptors are functional only if the three different NMDA subunit classes (NR-1, –2 and –3) are assembled in a specific homo-/heterotetrameric combination (Ulbrich and Isacoff 2008).

An assembly of the receptor complex takes place within the ER where the *N*-terminal domains of different iGluRs interact and recognise suitable partners (Ayalon and Stern-Bach 2001; Ayalon *et al.* 2005; Mah 2005; Penn *et al.* 2008). Quality control mechanisms guarantee a correct protein folding and receptor complex composition. During this step also auxiliary proteins are involved. The assembly within the ER could explain various difficulties when fusing tags to GLRs in plants. The marker could interfere with helper proteins and could impair the receptors ability to assemble in a native way causing retention of these fusion proteins within the ER lumen.

There is growing evidence that glutamate receptors in plants indeed exist as multimers. The use of antibodies against the conserved *C*-terminus of AtGLRs led to the detection of multimeric complexes (Turano *et al.* 2002). Also, methods like fluorescence resonance energy transfer (FRET) verified a homo- and heteromultimer formation of AtGLR3.2 and AtGLR3.4 at least when expressed transiently within tobacco leaf cells (Vincill *et al.* 2012, 2013). Additionally, observations using a method derived from the *yeast* two-hybrid (Y2H) system, called 'yeast mating-based split ubiquitin system', allowed the detection of homo-/heteromeric protein interactions between different AtGLRs at the plasma membrane (Price and Okumoto 2013). In this respect, AtGLR2.9, AtGLR3.2 and AtGLR3.4 appear to be crucial actors in mediating the contact of different glutamate receptors probably by an interaction of their *N*-terminal domains similar to iGluRs (Traynelis *et al.* 2010). Unlike in these receptors where only subunits of the same class can interact with each other, the actual formation of the receptor complex of AtGLRs seems to be independent of the three different clades found in *A. thaliana* (Dingledine *et al.* 1999; Price and Okumoto 2013).

The elementary composition of a single GLR as well as the whole receptor complex indicates not only a structural identity of animal and plant glutamate receptors. It further prompts a similar function in respect to ligand binding and ion conduction.

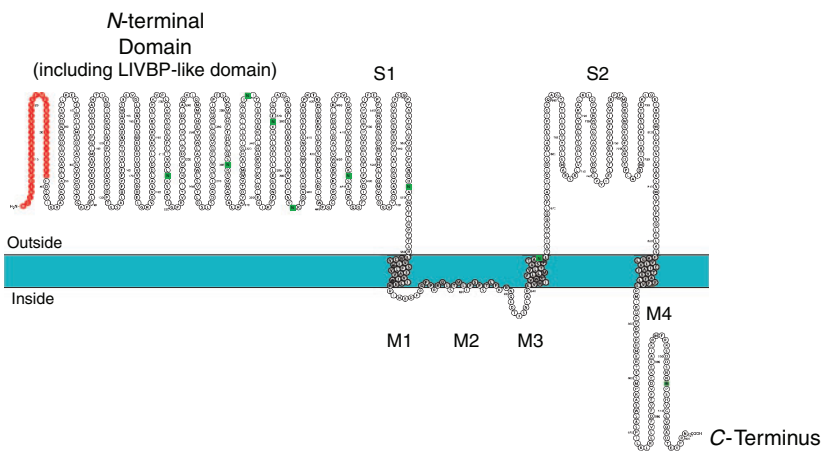

Fig. 1. Illustration of a theoretical incorporation of *Arabidopsis thaliana* glutamate receptor AtGLR3.7 into a lipid bilayer. The signal sequence within the amino acid sequence of the receptor is marked in red whereas possible glycosylation sites are marked in green. Transmembrane predictions were performed using Protter software (Omasits *et al.* 2014).

Therefore, basic knowledge about iGluRs can be transferred to the plant system.

Homology of domains and unique GLR structures

Animal iGluRs contain six essential domains and all of them are present in GLRs (Lam *et al*. 1998). It is assumed that the evolutionary assembly of the functional transmembrane domains and the ligand-binding sites took place before the divergence of animals and plants. The similarities between plant and animal glutamate receptors are very high when comparing both the whole receptor structure and the separate domains. The overall homology of GLRs and iGluRs is ~50–60% with a lower similarity in the M2 domain of the pore region (Chiu *et al*. 2002; Nagata 2004). Since this domain is responsible for the ion flux, the low sequence identity had led to speculations about the true GLR ion conductance capabilities. Variations within the pore region impede any attempts to draw exact conclusions from iGluRs to GLRs concerning their ion selectivity.

In contrast, the highest sequence identity exists within another domain of the pore region (M3) with a similarity up to 61% (Chiu *et al*. 1999; Kim *et al*. 2001). This strong homology could account for the important functionality of the region within the receptors. But also the ligand-binding domains S1 and S2 contain highly conserved residues (Chiu *et al*. 1999). When comparing amino acid sequences of *Arabidopsis* GLRs and their closest relative NMDA receptor GluN1 many amino acids are identical or at least very similar (Fig. 2).

Comparing the six domains among the 20 GLRs of *A. thaliana* itself, there is little difference within the four transmembrane domains. M1, M2 and M3 seem to be highly conserved within all three AtGLR clades, indicating identical ion selectivity (Chiu *et al*. 2002). In contrast, the ligand-binding sites S1 and S2 differ clearly between the three clades (Chiu *et al*. 2002). This fact would allow each clade or even each single receptor its own ligand-binding capacities for ligands like amino acids or structural-related molecules. Taking in account that every glutamate receptor complex consists of four GLRs this enables, in theory, an enormous number of unique receptor complexes each one with its distinct potential ligands. A perception of a multitude of different signals would be achievable and it could explain the already described involvements of GLRs in various physiological processes (see 'Physiological effects').

Besides the mentioned six conserved domains there is also evidence for unique structures in AtGLRs. A G protein-coupled receptor (GPCR)-like domain is present in most of the plant glutamate receptors. This could establish an evolutionary linkage between different GLRs in various species (Chiu *et al*. 1999; Turano *et al*. 2001). In addition, some of the AtGLRs contain also a long *N*-terminal sequence with a high level of similarity to both extracellular calcium sensors and glutamate/4-aminobutanoic acid (GABA) receptors (Turano *et al*. 2002; Nagata 2004).

Both domains could enhance potential receptor regulations in binding of several other ligands to these regions. Indeed, a fine-tuning of iGluRs by phosphorylation, palmitoylation, glycosylation and S-nitrosylation is affecting the receptors responsiveness (see Traynelis *et al*. 2010). Since at least phosphorylations and glycosylations are likely to occur also in plant glutamate receptors, the localisation and activity of GLRs is probably in the same way affected by this means as it is by ligands acting as modulator on alternative binding sites.

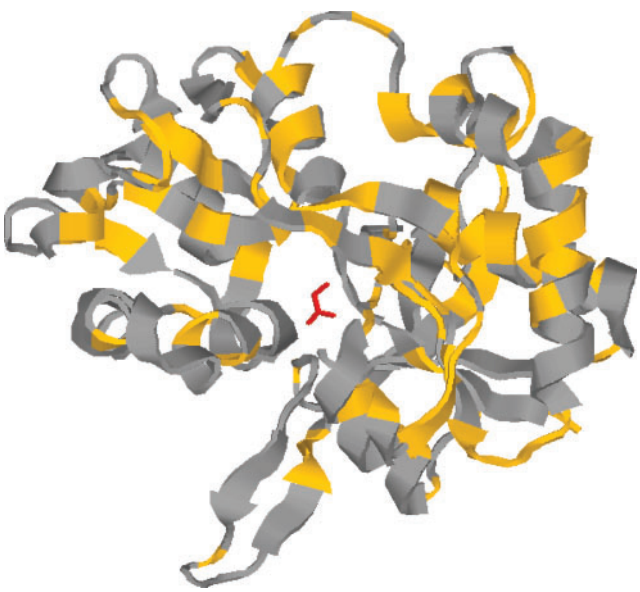

Fig. 2. Crystal structure of the ligand binding domain of glutamate receptor Grin1 of *rattus norvegicus* in complex with glycine. Amino acids that are identical/similar to the amino acid sequence *of Arabidopsis thaliana* glutamate receptor AtGLR3.7 are highlighted in yellow. The glutamate receptor ligand glycine is shown in red. The adapted crystal structure is based on an already described structure in Furukawa and Gouaux (2003). Displayed complex modified by RasMol software (Sayle and Milner-White 1995).

Ligand binding sites

Besides the conserved membrane spanning domains M1–M4, GLRs contain two different ligand-binding regions that both are located on the external site of the membranes (Lam *et al*. 1998). One ligand-binding domain is composed of the two putative ligand-binding sites S1 and S2 (Lam *et al*. 1998). These sites form a lysine/arginine/ornithine-binding protein (LAOBP)-like domain with a homology to the periplasmic binding protein-like II superfamily (Acher and Bertrand 2005). The LAOBP-like domain can be found also in iGluRs and it is supposed to bind glutamate (Paas 1998). The second ligand-binding domain is located near the *N*-terminus and exhibits a similarity to a leucine/isoleucine/valine-binding protein (LIVBP) domain. This second domain could fulfil a modulating function of the receptor complex in binding a second molecule. Potential additional ligands are either other amino acids or a completely different agent (Acher and Bertrand 2005).

In both cases, the binding of the ligand is accomplished by a 'Venus flytrap mechanism' similar to that one in NMDA receptors (Felder *et al*. 1999; Acher and Bertrand 2005). In this receptor class the binding of the ligand takes place between two LIVBP-like domains and their responsiveness to either glutamate or glycine is determined by the composition of the receptor complex (Dubos *et al*. 2003; Acher and Bertrand 2005; Traynelis *et al*. 2010). Similarly, in GLRs, an open structure

of the LIVBP-like domain enables a ligand to bind in its centre. The attachment of the molecule leads to conformational changes that cause a closing of the ligand-binding sites (Acher and Bertrand 2005). This, in turn, evokes alterations near the receptor pore region and allows a passage/blockade of ions.

Distinct amino acids within the S1 and S2 sites are required for a proper ligand binding and receptor function. For AtGLR1.4, it could be demonstrated that alterations of the amino acids D499, T501 and R506 result in a strong reduction or even a loss of function of ligand-gated cation currents when expressed in *Xenopus* oocytes (Tapken *et al.* 2013). The fact that these three amino acids are conserved in all AtGLRs clearly underlines a strong sensitivity of GLRs against amino acids. In the particular case of AtGLR1.4, the agonist profile comprises amino acids with bulky, hydrophobic side chains (Tapken *et al.* 2013). The hypothesis of amino acids as the true ligands for plant glutamate receptors is supported by the fact that most of the active agents on GLRs are related to certain amino acids. Whereas common iGluR agonists like serotonin, melatonin, dopamine, acetycholine or indole-3-acetic acid (IAA) seems to be ineffective on GLRs. The primary functional amino acid groups (α-amino/β-carboxyl) are probably required for a binding at GLRs. This feature is known for animal iGluRs in which ligand-binding site S1 seems to be responsible for the recognition of α-amino and β-carboxyl groups (Traynelis *et al.* 2010; Tapken *et al.* 2013).

A way to modify the receptor's binding capacities could be through glycosylations of amino acid side chains within the *N*-terminal region. The glycosylation status of this part affects receptor trafficking, desensitisation status, maximal conductance as well as the ability of ligands to bind at the respective sites at least in iGluRs (Standley and Baudry 2000). Likewise, all AtGLRs contain potential glycosylation sites that can be found predominantly within their *N*-terminus (see Fig. 1). Therefore, glycosylation events are highly likely to occur also on plant glutamate receptors and could affect their function in a similar way as is does within iGluRs.

Ligands

It is very likely that working plant GLRs are capable of binding different ligands depending on the composition of the four subunit-comprising receptor complex. Having in mind that 20 GLRs exist in *A. thaliana* while each subunit is capable of binding two (particular) ligands, the number of possibilities for variant receptor complex compositions is enormous and helps to explain the involvement of GLRs in many different physiological aspects (see 'Physiological effects').

It is noteworthy that classical iGluR agonists like NMDA, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) or (2S,3S,4S)-3-(carboxymethyl)-4-prop-1-en-2-ylpyrrolidine-2-carboxylic acid (kainate) do not have any effect on GLRs in *Arabidopsis* and they also seem to be completely absent in plants (Vatsa *et al.* 2011). There are only two published studies in which these classical agonists were effective. A GABA treatment in tobacco culture cells caused elevations of the cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$) and NMDA evoked action potentials (AP) in a liverwort (Krol *et al.* 2007; Vatsa *et al.* 2011). However, both cases represent either an unusual set-up or a quite distant model plant and conclusions should be drawn carefully.

It is probable that these agents are ineffective since they are not amino acids and do not exhibit an amino acid-related structure (see 'Ligand binding sites').

Agonists

The true agonists of GLRs are not fully elucidated even today. In spite of significant similarities between iGluRs and GLR, there are apparent deviations within the ligand-binding sites between these two receptor types. The amino acid glutamate is often seen as the main agonist of GLRs due to their structural analogy to animal iGluRs. However, the first algorithm predictions for GLRs contested this assertion. Earlier, Dubos *et al.* (2003) proposed glycine instead of glutamate as the main agonist for most of the AtGLRs. Only AtGLR1.1 ought to be a real glutamate-binding subunit. In iGluRs the amino acid Thr655 is highly conserved and seems to be crucial for glutamate binding whereas GLRs of *A. thaliana* possess the structurally different amino acid phenylalanine at this position (Dubos *et al.* 2003). It could be that Phe665 in *Arabidopsis* had originally been threonine, too, but finally became replaced in almost all AtGLRs (Dubos *et al.* 2003). Due to the bulky structure of phenylalanine, Dubos *et al.* (2003) excluded glutamate as a natural ligand for GLRs.

Nonetheless, a multitude of potential GLR agonists has emerged in the meantime. As mentioned before, an increasing number of findings points to a mostly amino acid-gated mechanism as can be seen by reported agonists/antagonists (Table 1). Membrane depolarisations as well as Ca^{2+} influxes are observable not only by the application of glutamate and glycine but also asparagine, serine, alanine, cysteine, methionine and glutathione (Dennison and Spalding 2000; Qi *et al.* 2006; Stephens *et al.* 2008; Vincill *et al.* 2012; Li *et al.* 2013*a*; Tapken *et al.* 2013). AtGLR3.3 may be essential for the perception of glutamate since its knockout causes insensitivity to this amino acid while AtGLR3.4 is probably necessary to respond to serine and alanine treatments (Stephens *et al.* 2008).

Because of its α-amino and β-carboxyl groups, glutathione in its reduced form is in accordance with the ligand-binding pattern so far observed. However, its oxidised form could interfere with the native structure of glutamate receptors by creating disulfide bonds. In NMDA receptors this formation inhibits cation currents and helps to regulate the receptor's function depending on the cellular redox status (Choi and Lipton 2000). There is still some debate over whether the iGluR agonist β-methylamino-L-alanine (BMAA) acts as an agonist or antagonist considering its counteraction of some glutamate-mediated effects (Brenner *et al.* 2009). In *A. thaliana*, BMAA causes a rapid membrane depolarisation and its further effects are indicative for a role as a possible GLR ligand (Brenner *et al.* 2000, 2009). Besides BMAA, there are assertions based on *in silico* analysis of different AtGLR members that kanamycin and related polyamines could belong to a group of GLR agonists (Dubos *et al.* 2005). At least the pore region of AtGLR1.1 allows an intercalation of polyamines (Tapken and Hollmann 2008). Following this study, a binding within this domain would block or alter inward rectifying currents similar to what is observed in

Table 1. Overview about tested chemicals as potential agonists, antagonists or blocker of glutamate receptors of *Arabidopsis thaliana*
Effects, commonly-used concentration and target of the respective agent depending on findings in the corresponding publications

Tested chemical	Concentration(s) (mM)	Involved GLR(s)	Reference(s)
Potential agonist			
L-Glutamate	0.01; 0.02; 0.05; 0.1; 0.25; 0.5; 1; 2; 3; 5; 10; 15	AtGLR3.3 RsGluR	Brenner *et al*. (2000); Dennison and Spalding (2000); Dubos *et al*. (2003); Kang and Turano (2003); Sivaguru *et al*. (2003); Demidchik *et al*. (2004); Meyerhoff *et al*. (2005); Kang *et al*. (2006); Qi *et al*. (2006); Walch-Liu (2006); Krol *et al*. (2007); Stephens *et al*. (2008); Teardo *et al*. (2010); Kwaaitaal *et al*. (2011); Vatsa *et al*. (2011); Tapken *et al*. (2013)
Glycine	0.01; 0.1; 1; 5; 10; 15	AtGLR3.3 AtGLR3.4	Dubos *et al*. (2003); Meyerhoff *et al*. (2005); Qi *et al*. (2006); Krol *et al*. (2007); Stephens *et al*. (2008); Teardo *et al*. (2010); Michard *et al*. (2011); Vincill *et al*. (2012)
Asparagine	0.1; 1; 3; 10	AtGLR1.4 AtGLR3.3 AtGLR3.4	Qi *et al*. (2006); Stephens *et al*. (2008); Vincill *et al*. (2012); Tapken *et al*. (2013)
Alanine	0.1; 1; 10	AtGLR3.3 AtGLR3.4	Meyerhoff *et al*. (2005); Qi *et al*. (2006); Stephens *et al*. (2008)
L-Serine	0.1; 1; 10	AtGLR3.3 AtGLR3.4	Qi *et al*. (2006); Stephens *et al*. (2008); Vincill *et al*. (2012)
D-Serine	0.1; 1; 5		Michard *et al*. (2011)
Cysteine	0.1; 1; 10	AtGLR3.3 AtGLR3.4	Qi *et al*. (2006); Stephens *et al*. (2008); Li *et al*. (2013*a*)
Glutamine	10		Kang and Turano (2003)
Methionine	1	AtGLR1.4	Tapken *et al*. (2013)
Tryptophan	1	AtGLR1.4	Tapken *et al*. (2013)
Tyrosine	1	AtGLR1.4	Tapken *et al*. (2013)
Threonine	1	AtGLR1.4	Tapken *et al*. (2013)
Leucine	1	AtGLR1.4	Tapken *et al*. (2013)
Phenylalanine	1	AtGLR1.4	Tapken *et al*. (2013)
Glutathione	0.1; 1	AtGLR3.3	Qi *et al*. (2006) Li *et al*. (2013*a*)
BMAA	0.02; 0.05; 0.1; 0.2		Brenner *et al*. (2000); Kang and Turano (2003); Brenner *et al*. (2009)
Kynurenic acid	1		Kwaaitaal *et al*. (2011)
(NMDA)	0.1; 10		Sivaguru *et al*. (2003); Krol *et al*. (2007)
Potential antagonist			
DNQX(selective non-NMDA receptor antagonist; binds in pore)	0.1; 0.25; 0.5; 1	AtGLR3.3 AtGLR1.4	Lam *et al*. (1998); Kang and Turano (2003); Kang *et al*. (2004); Meyerhoff *et al*. (2005); Krol *et al*. (2007); Teardo *et al*. (2010); Michard *et al*. (2011); Vatsa *et al*. (2011); Li *et al*. (2013*a*); Tapken *et al*. (2013)
AP-5(competitive NMDA receptor antagonist for L-glutamate binding site)	0.05; 0.1; 1; 2	AtGLR3.3	Sivaguru *et al*. (2003); Krol *et al*. (2007); Kwaaitaal *et al*. (2011); Michard *et al*. (2011); Vatsa *et al*. (2011); Li *et al*. (2013*a*)
CNQX(potent AMPA/kainate antagonist)	0.25; 0.5; 1	AtGLR1.4	Meyerhoff *et al*. (2005); Michard *et al*. (2011); Tapken *et al*. (2013)
MK-801(non-competitive NMDA receptor open channel blocker)	0.35; 0.1	AtGLR1.4	Vatsa *et al*. (2011); Tapken *et al*. (2013)
MNQX	0.5		Meyerhoff *et al*. (2005)
AP-7(competitive NMDA receptor antagonist on L-glutamate binding site)	1		Kwaaitaal *et al*. (2011)
Kynurenic acid(non-selective NMDA receptor antagonist on glycine binding site)	1		Kwaaitaal *et al*. (2011)
Memantine(NMDA receptor antagonist)	1		Vatsa *et al*. (2011)
Philanthotoxin(AMPA/kainate receptor blocker)	0.1	AtGLR1.4	Tapken *et al*. (2013)

(*continued next page*)

Table 1. (*continued*)

Tested chemical	Concentration(s) (mM)	Involved GLR(s)	Reference(s)
Agonists without effect			
Aspartate	1; 5; 10; 50		Dennison and Spalding (2000); Dubos *et al.* (2003); Sivaguru *et al.* (2003); Demidchik *et al.* (2004); Meyerhoff *et al.* (2005); Krol *et al.* (2007); Walch-Liu *et al.* (2006)
D-Glutamate	0.05; 1	AtGLR3.3	Dennison and Spalding (2000); Qi *et al.* (2006); Walch-Liu *et al.* (2006)
GABA	0.05; 1; 10	AtGLR3.3	Meyerhoff *et al.* (2005); Qi *et al.* (2006); Walch-Liu *et al.* (2006)
NMDA	1	AtGLR3.3	Dennison and Spalding (2000); Qi *et al.* (2006)
Arginine	1		Dennison and Spalding (2000); Demidchik *et al.* (2004)
L-Alanine	0.1; 1; 10	AtGLR3.4	Dubos *et al.* (2003); Vincill *et al.* (2012)
L-Glutamate	0.1; 1	AtGLR3.4	Michard *et al.* (2011); Vincill *et al.* (2012)
AMPA	1		Dennison and Spalding (2000)
D-Alanine	1	AtGLR3.3	Qi *et al.* (2006)
Glutamine	10		Meyerhoff *et al.* (2005)
Glycine	0.05		Walch-Liu *et al.* (2006)
Tryptophan	0.05		Walch-Liu *et al.* (2006)
D-Serine	1	AtGLR3.3	Qi *et al.* (2006)
L-Serine	1		Michard *et al.* (2011)
Cysteine	0.1; 1	AtGLR3.4	Vincill *et al.* (2012)
Phenylalanine	0.1; 1	AtGLR3.4	Vincill *et al.* (2012)
All amino acids except Glu, Gly, Ser, Cys, Ala, Asn	1	AtGLR3.3	Qi *et al.* (2006)

AMPA/kainate receptors. It seems conceivable to assume a binding of kanamycin within the pore loop rather than at the ligand-binding domains. The so caused rectified inward currents would only mimic the agonist-induced activity of GLRs. Therefore, kanamycin and structural similar molecules should not be considered as agonists to glutamate receptors in plants.

On the other hand, it is supposed that different GLRs are targeted or activated by distinct amino acids. In this respect, the amino acids do not induce Ca^{2+} currents equally but in a hierarchical manner. The complexity of these precise interactions of AtGLR agonists was revealed by Stephens *et al.* (2008). In this particular study, a model was developed in which three receptor complex classes display distinct susceptibility patterns against specific ligands. These findings are in accordance with the assumption that functional GLRs are formed by four subunits and possess their own responsiveness due to their unique composition (Dubos *et al.* 2003; Acher and Bertrand 2005). Also, a competitive antagonism of distinct amino acids against others seems to exist in GLRs. A thorough investigation of AtGLR1.4 revealed a counteraction of different amino acids against its strongest agonist methionine (Tapken *et al.* 2013). This finding, in turn, counts for a broader ligand-binding profile of individual AtGLRs in which different types of amino acids can bind to a single receptor depending on the amino acid structure and its positive or negative charges (Tapken *et al.* 2013).

Antagonists

The use of appropriate antagonists is essential to cross-validate the effects exerted by glutamate and other ligands on GLRs. When using common iGluR antagonists it seems that some of them are functioning in plants, too. The most used agents are 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 2-amino-5-phosphonopentanoic acid (AP-5). The inhibiting effect of DNQX on all AtGLRs is supposed to rely on the attachment inside of the ligand-binding sites whereas AP-5 is probably competing with the natural ligand at the L-glutamate binding site (Dubos *et al.* 2003). Other commonly used antagonists and iGluR blockers include MK-801, memantine, 5,7-dinitro-1,4-dihydro-2,3-quinoxalinedione (MNQX) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Meyerhoff *et al.* 2005; Vatsa *et al.* 2011). The use of broader ion channel blockers like La^{3+} and Gd^{3+} can be helpful to verify a Ca^{2+} involvement but bears a lack of specificity since almost all important ion fluxes across membranes are impeded.

Ion conduction

GLRs are mainly considered as non-selective cation channels (Tapken and Hollmann 2008; Tapken *et al.* 2013). The passage of cations such as Ca^{2+}, K^+ and Na^+ is possible through the so called 'three-plus-one' motif. This motif is composed of three trans-membrane domains (M1, M3, M4) plus an only half of the membrane spanning domain (M2) (Fig. 1) (Lam *et al.* 1998). Together, M1-M4 form a 'pore loop', which is seen as the actual place where ions can pass through. This particular structure can be found in several ion channels, such as voltage-gated $K^+/Na^+/Ca^{2+}$ channels, cyclic nucleotide-gated channels or inward rectifier K^+ channels (Chiu *et al.* 1999). We note that one of the pore forming domains (M2) is a place where RNA editing occurs (Chiu *et al.* 1999). RNA editing in iGluRs takes place at the homologous M2 domain of AMPA and kainate receptors. Here, the editing leads to a changed Ca^{2+} permeability and sensitivity to polyamine channel blockers

through a conversion of glutamine into arginine within the ion selectivity QRN site (Dingledine *et al*. 1999; Traynelis *et al*. 2010). If this is the case in plants too, then it is possible that one single GLR possesses different ion selectivities depending on the tissue in which it is translated or the developmental stage of the plant. At the end, the final ion conductivity and electrical properties of the active receptor complex within excitable plant cell membranes are determined by a combination of various AtGLRs.

The true ion permeability of a GLR cannot be assigned by only comparing the amino acid sequences of plant and animal glutamate receptors. The plant GLR pore region features some characteristic deviations in its sequence. Considering that a single amino acid exchange in an AMPA receptor subunit leads to a loss of its Ca^{2+} permeability (Nagata 2004), it is feasible to expect altered ion selectivity in plants. Furthermore, mutations within the pore loop region of AtGLRs can cause a severe alteration of outward/inward rectifying currents, as shown by Tapken and Hollmann (2008). These findings indicate the possibility of a modulation of currents by single amino acid exchanges. The idea of a plant-specific ion conductance is supported by the fact that AtGLRs are missing ion selectivity motives such as a QRN-site that is common in all iGluRs (Nagata 2004). A spot like this is not only essential for the Ca^{2+} selectivity/permeability but also for an Mg^{2+}-caused blockage of the receptors (Nagata 2004).

In contrast, there is profound evidence for ion conductions via AtGLRs at least in heterologous expression systems. Studies aiming to elucidate the exact ions involved in currents induced by glutamate and other potential GLR agonists point to cation fluxes with a clear preference for Ca^{2+}. The heterologous expressions of AtGLR1.4, AtGLR 3.4 or AtGLR3.7 in *Xenopus* oocytes lead to constitutive currents of Ca^{2+}, Na^{+}, K^{+} and Ba^{+} (Roy *et al*. 2008; Tapken *et al*. 2013). Whereas the fluxes mediated by AtGLR1.4 were sensitive to different amino acids, the currents of AtGLR3.4 and AtGLR3.7 were voltage-independent and did not respond to any kind of known GLR agonists. Calcium fluxes were also observed for heterologous expressed AtGLR3.2 and AtGLR3.4 in HEK cells (Vincill *et al*. 2012, 2013).

Another work verified a gating mechanism of Ca^{2+} fluxes by chimeric glutamate receptor constructs. For this study only the pore region and its adjacent loops of 17 different AtGLRs were incorporated into a rat kainate receptor subunit (GluK2) or a rat AMPA receptor subunit (GluA1) (Tapken and Hollmann 2008). In this way a circumvention of the frequently encountered difficulties concerning the assembly and function of an AtGLR receptor complex was achieved in order to concentrate only on the ion pore domain. The heterologous expression of these chimeras in *Xenopus* oocytes leads to measurable Ca^{2+}, Na^{+} and K^{+} currents. When observing the reversal potentials of the glutamate-induced fluxes, these currents exhibit a distinct behaviour. While Na^{+}/K^{+} fluxes are unaffected, the permeability of the plasma membrane to Ca^{2+} seems to be elevated. Furthermore, the calcium currents of two of these receptor chimeras (AtGLR1.1-GluK2/-1 and AtGLR1.4-GluK2/-1) were inducible by the iGluR agonist kainate whereas the AtGLR1.4-GluK2/-1 chimera was activated only by glutamate. Complete inhibition of the kainate-induced Ca^{2+} fluxes in AtGLR1.1-GluA1 was achieved by the ion channel blocker La^{3+}, whereas the AMPA/kainate receptor blocker 1-naphtylacetylspermine (NASP) and the NMDA receptor blocker [5R,10S]-[+]-5-methyl-10,11-dihydro-5*H*-dibenzo [*a*,*d*]cyclohepten-5,10-imine (MK-801) led to only a partial inhibition (Tapken and Hollmann 2008). The findings of the agonism and antagonism of these agents confirm the functionality of the chimeras since the ligand-binding sites originate from AMPA and kainate receptors and comprise their activation/inactivation features. It further indicates a similar way of gating, which means that the conformational changes exerted by the ligand-binding domains of the animal iGluRs have also an effect on the pore region of the plant GLRs. For animal iGluRs, it was demonstrated that the *N*-terminal domain controls the open/close state of the pore region (see Traynelis *et al*. 2010). This domain is susceptible to manifold modifications that influence the desensitisation and activation properties of the whole receptor complex. Since in the chimera experiments this domain is derived from animal AMPA or kainate receptors, their characteristics could differ from the actual plant GLRs. In addition to that, the functionality of the chimeric receptors depends presumably mainly on these *N*-terminal domains from mammalian iGluRs. In this respect, the study by Tapken and Hollmann (2008) should be taken as a proof for the ion conducting capacities of GLRs and an indication for pronounced structural similarities in this domain.

A noteworthy finding of this study concerns the induced currents itself. Only 2 of the 17 AtGLR pore regions were functional and even these did not have any glutamate- or kainate-regulated activity. No inducible Ca^{2+} fluxes were detected until the addition of the AMPA receptor regulatory protein 'TARP γ-2' (Tapken and Hollmann 2008). This observation could help to explain why electrophysiological studies using heterologous expressions are still hard to conduct and investigations often fail (Vincill *et al*. 2013; Teardo *et al*. 2015). It appears that the GLR complex, which contains several different AtGLR subunits, is highly sophisticated and needs auxiliary proteins and an accurate composition to work correctly.

However, caution should be taken when employing heterologous expressions of ion channels. The usage of these systems entails some factors that diminish their reliability due to risks of misfolding or a wrong processing of the protein. Post-translational modifications such as glycosylation and cleavage of precursor forms are most probably affected (Rai and Padh 2001). Furthermore, endogenous ion currents derived from the host system can have an influence on the actual measurements and need robust controls (see Varghese *et al*. 2006; Tammaro *et al*. 2009).

Receptor activation and modulation

The mechanisms underlying GLR functions are supposed to be comparable to other receptors and channels. In general, three different states of a receptor can be distinguished. In the active state the receptor is responsive to binding of ligands. After the ligand binding, there is most often only a short period in which ion fluxes across the membrane can occur until the receptor changes to a desensitised or inactive state in which the ligand is still bound but no other actions can take place. Under these conditions, ion currents are typically terminated. In order to restore the sensitivity to a specific stimulus, the receptor complex must either release its ligand directly at the plasma

membrane or the receptor needs to be incorporated into the cell for degradation or recycling. Inducible *de novo* synthesis would be necessary if the receptor is targeted for degradation.

In their active state, GLRs respond to glutamate, glycine and other agonists and are susceptible to antagonists like DNQX or unspecific ion channel blockers such as La^{3+} or Gd^{3+} (Meyerhoff *et al*. 2005). The acutal ligand-binding causes a long-lasting insensitivity to repeated ligand treatments due to processes close to the active domains, i.e. conformational changes (Stephens *et al*. 2008). It is not completely understood how sensitivity is maintained during a GLR-mediated signalling so far. Some evidence indicates a *de novo* biosynthesis since the translation inhibitor cycloheximide (CHX) prevents a restoration of the sensitivity to GLR agonists and antagonists (Meyerhoff *et al*. 2005). Additionally, an enhanced GLR gene expression is detectable when specific external stimuli are applied (Meyerhoff *et al*. 2005). Both observations argue for an inducible and non-constitutive receptor system.

In animals, NMDA/kainate receptors need to be degraded to control their exact subcellular localisation and precise numbers to function properly (Kato *et al*. 2005; Salinas *et al*. 2006). It is already known, that the phosphorylation status of the *C*-terminus of iGluRs affects receptor trafficking, plasma membrane insertion, subcellular localisation and recycling (Traynelis *et al*. 2010). The *C*-terminus of iGluRs furthermore contains docking motifs for intracellular binding proteins involved in signalling events including targeting for protein degradation (Traynelis *et al*. 2010). Similar mechanisms have to be expected also in plants.

Support for the necessity of an appropriate GLR degradation originates from an *Arabidopsis* mutant line (bim409) in which a subunit of the 26S proteasome is affected (Brenner *et al*. 2009). Since the 26S proteasome is involved in degradation of proteins, a mutation could have severe impacts on the signalling systems. Actually, the bim409 mutant line exhibits a reduced sensitivity to the GLR ligand BMAA and its mediated effects (Brenner *et al*. 2000; Teardo *et al*. 2010). It is possible that the responsible GLRs is altered by insufficient receptor degradation and this, in turn, causes a reduced signal perception against BMAA.

Both in the receptor recycling and receptor degradation, there is a demand for a proper targeting of the proteins. Phosphorylation of proteins is one of the most common strategies for this purpose. As to that, 14-3-3 proteins are important mediators for discriminating and processing such events since they recognise phosphorylated/dephosphorylated proteins and promote an interaction with other kinases or phosphatases (Fu *et al*. 2000). iGluRs and the related metabotropic glutamate receptors (mGluRs) interact with 14-3-3 proteins (Angrand *et al*. 2006). Regarding the AtGLRs, 16 out of 20 AtGLRs contain 14-3-3 binding sites and for at least five of these receptors (AtGLR1.2, 2.1, 2.9, 3.4 and AtGLR3.7) an interaction with 14-3-3 proteins is affirmed (Chang *et al*. 2009).

Taking together what is known about related glutamate receptors in other species and research conducted on plant GLRs, it would be unexpected to find GLRs as static ion channels instead of highly regulated members of signalling pathways. Further experiments aiming at the investigation of *C*-terminal phosphorylation events or the determination of glycosylation patterns at the *N*-terminus would lay the basis for a better understanding how these ion channels are regulated on the receptor itself.

Subcellular localisation

Sequence analysis provided the first information about the AtGLR localisations. Growing evidence supports a theory in which glutamate receptors in plants are transported to and incorporated in membranes. Signal peptides within the *N*-terminus of most of the GLRs indicate at least an entering of the secretory pathway. Therefore, integration into the plasma membrane seems to be likely (Lam *et al*. 1998; Chiu *et al*. 1999; Nagata 2004; Teardo *et al*. 2010). Besides the signal sequences for the secretory pathway, mitochondrial and chloroplast targeting sequences can be found (Teardo *et al*. 2010, 2011). When looking at clade III in *A. thaliana*, four of seven receptors (AtGLR3.1, 3.2, 3.6 and AtGLR3.7) are supposed to enter the secretory pathway while the three others (AtGLR3.3, 3.4 and 3.5) contain multiple targeting sequences (Teardo *et al*. 2010, 2011). Also, GLRs from other plant species than *Arabidopsis* include signal peptides for secretion (i.e. small radish (RsGluR), rice (OsGLR3.1)). Their GFP-tagged versions were found to enter the secretory pathway or localise at the plasma membrane (Kang *et al*. 2006; Li *et al*. 2006).

In the beginning it was reported that cDNAs of plant glutamate receptors display a toxicity in *Escherichia coli* and eukaryotic cells that led to truncated or mis-spliced mRNA versions of some GLRs (Davenport 2002). Because of these initial problems and difficulties in constructing GFP fusion proteins of GLRs, an alternative way was chosen to detect them on a subcellular level. The use of antibodies against conserved and non-conserved domains of members of AtGLR clade III was capable in revealing their location within membranes of different plant cells (Turano *et al*. 2002). By this method, some GLRs were found not only as plasma membrane located proteins but also integration into the inner chloroplast membranes could be detected (Teardo *et al*. 2011).

In this way, glutamate receptors of plants could participate additionally in the intracellular signal transduction by releasing Ca^{2+} from internal stores such as the ER. It is possible that the ER localisation of some GLRs (see Li *et al*. 2006; Singh *et al*. 2014) is a native feature and these receptors act as genuine ER receptors with a role in this Ca^{2+}-enriched cellular compartment.

In 2005, Meyerhoff *et al*. (2005) showed for the first time a localisation of a GFP-tagged AtGLR at the plasma membrane of onion epidermal cells. Since then, fluorescent tags revealed the existence of different glutamate receptors in the plasma membrane, the membrane of plastids and in stromules of *Arabidopsis* (Teardo *et al*. 2011; Vincill *et al*. 2012, 2013; Tapken *et al*. 2013). The plastid-derived signals from AtGLR3.4-YFP in *Arabidopsis* could also be detected in transient expressions in tobacco leaf cells where this receptor seems to localise in the outer or inner envelope of plastids but not within thylakoids (Teardo *et al*. 2011). The presence of GFP fusion proteins of AtGLR3.2, 3.3 and AtGLR3.4 in all cell types of the *Arabidopsis* root growth zone as well as an accumulation in the plasma membrane of sieve plates confirm previous results of an enhanced GLR gene expression in the phloem of the mature

region of the root (Chiu *et al*. 2002; Turano *et al*. 2002; Vincill *et al*. 2013).

Noteworthy, a recent study by Teardo *et al*. (2015) demonstrates the existence of alternative mRNA splice variants of AtGLRs and their impact on the integration of the same receptor in different membranes. It could be shown that AtGLR3.5 contains an *N*-terminal signal sequence for mitochondria that is removed in another isoform. Actually, this leads to a localisation of one isoform at the inner mitochondrial membrane where its *C*-terminus faces the matrix and its ligand-binding sites the cytosol. The other isoform is situated in chloroplasts (Teardo *et al*. 2015). The phenomenon of alternative splicing in GLRs could amplify the flexibility of some glutamate receptors in respect to potential sites of action and signal modulations.

Expression analysis

Soon after their discovery, it became clear that all GLRs are expressed in *Arabidopsis*, with at least three of them (AtGLR2.5, AtGLR3.4 and AtGLR3.5) occurring as mRNA splice variants (Chiu *et al*. 2002; Meyerhoff *et al*. 2005; Teardo *et al*. 2015). The existence of different spliced forms of mRNAs allows an even broader spectrum of involvements and functions for these receptors by multiplying significantly possible receptor complex combinations (see 'Molecular structure').

Genes of clade I and III are expressed in all tissues throughout the plant and the highest expression levels were observed within the root (Chiu *et al.* 2002). The only exceptions are those of AtGLR3.2 and AtGLR3.4, which are not expressed in siliques (Chiu *et al.* 2002).

In contrast, the expression of clade II genes is quite different. Five out of nine genes of this clade are expressed only in roots (AtGLR2.1, –2.2, –2.3, –2.6 and AtGLR2.9), one is expressed in roots and siliques (AtGLR2.4), two are expressed in all organs but flowers (AtGLR2.7 and AtGLR2.8) (Chiu *et al.* 2002; Gilliham *et al.* 2006). Only one member of clade II (AtGLR2.5) is expressed within the whole plant (Chiu *et al.* 2002). There is a possibility that the expression restriction of AtGLR2.1–2.3, 2.6, 2.9 in 8-week-old plants classifies them as a functional class (Chiu *et al*. 2002), or it just underlines their reduced importance because they can be substituted by other GLRs.

Thanks to a thorough investigation concerning the expression of AtGLRs on a single cell level by Roy *et al*. (2008), it could be confirmed that all 20 AtGLRs are expressed in the *Arabidopsis* roots, when a loose restriction of AtGLR clade II to the roots was reasserted. Furthermore, there are 16–18 genes expressed in stems, petioles and leaves but a continuing downregulation of members of clade II occurs during plant development with either very low levels in leaves (AtGLR2.2 and AtGLR2.3) or even a complete expression stop as found for AtGLR2.1, 2.4, 2.6, 2.9 (Roy *et al*. 2008). Another even more important result in this study attests a simultaneous expression of five to six GLRs in one cell. Interestingly, the expression of a specific GLR shows high variations at the same cell type in different seedlings but displays higher similarities between different cell types within the same plant (Roy *et al*. 2008). This observation could mean that different sets of specific AtGLRs are activated during plant development in each plant individually. So either the individuals are actually more individual than expected, or there is a high degree of redundancy and functional overlap between the 20 AtGLRs.

The usage of the reporter β-glucuronidase (GUS) in *Arabidopsis* allows a closer look on the expression of the receptors during development. First AtGLR-GUS expressions can be observed five days after germination within the vascular tissue of cotyledons (Kim *et al.* 2001; Chiu *et al*. 2002; Turano *et al.* 2002). Later, there is an expression in all other organs like roots, flowers and siliques (Chiu *et al*. 2002; Gilliham *et al.* 2006). The highest expression levels were scored within the vasculature and its associated tissues (Chiu *et al*. 2002; Meyerhoff *et al.* 2005; Cho *et al.* 2009). Strong expression in cells that are specialised for water and nutrition transfer like the funiculus and within developing seeds could indicate a function of these receptors in the perception of amino acids combined with a role in mediating their transport as well as further processes downstream. However, most of the information gathered so far points to a clear involvement in signalling events within the plant.

The following selection of GUS assays and mRNA expression studies conducted on various AtGLRs gives an overview about their occurrence in different tissues and developmental stages. It furthermore points to an intensified expression of AtGLRs, mainly of clade III, within the xylem and phloem. The focus of the GLR gene expression in this part of the plant could enclose a further function of this tissue as a kind of 'signal transduction highway'. A much faster communication between distal parts of the organism could be feasible due the uniform structure and the relatively large and therefore capacitive cells of the vasculature.

Expression of selected AtGLRs

AtGLR1.1

First GUS detection can be determined in stipules and the collette region of 7-day-old *Arabidopsis* seedlings. After 3–4 weeks, the expression is expanded to the margins of leaves and all cell types of lateral roots except the root tip. Also, a weak and temporal expression in reproductive organs such as siliques and flowers can be observed (Chiu *et al*. 2002).

AtGLR2.1

An early expression, three days after germination, is detected within all cell types of the radical root including root hairs. But the root tip does not show any expression of this receptor. After five days AtGLR2.1 is expressed also is in the shoot (stipules). Similar to AtGLR1.1 a weak expression in reproductive organs occurs but much stronger in anthers and young ovules (Chiu *et al*. 2002).

AtGLR2.8

Like AtGLR2.1 also AtGLR2.8 is expressed not only in the roots but also in the shoots. An expression in leaves with a strong GUS straining can be observed around vascular bundles, and there seems to be an increased expression in leaves in the senescence stage (Gilliham *et al.* 2006).

AtGLR3.1

The AtGLR3.1-GUS line displays a strong staining of vascular tissue in roots, stems and leaves as well as in guard cells where its expression is higher than in the surrounding mesophyll cells (Cho *et al.* 2009). An involvement in stomatal regulations is very likely since a knockout of this receptor, as well as another member of clade III (AtGLR3.4), causes impairments in stomatal closing and lower photosynthetic yields (see 'Physiological effects').

AtGLR3.2

This glutamate receptor could participate in early plant developments but also during the inflorescent stage considering an upregulation of the gene (Turano *et al.* 2002). In general, the mRNA level is rather low in roots, leaves, flowers and siliques compared with its presence in floral stalks and bolts (Turano *et al.* 2002). AtGLR3.2 is predominantly found in the centre of developing ovules in the floral buds and in pollen grains (Turano *et al.* 2002): both indicate a possible role in the regulation of plant development at the reproductive stage.

Furthermore, a steady expression within the leaves is characterised by intensified staining of the vascular tissues (Turano *et al.* 2002). We note that the expression of AtGLR3.2 seems to be focussed on vascular tissues and adjacent conducting vessels throughout the plant including stems, leaves, flowers and roots (Kim *et al.* 2001; Turano *et al.* 2002). There is a probability for an activity within the phloem, especially the protophloem, of the root since the mRNA expression level in this tissue is much higher than in its neighbouring cells (Brady *et al.* 2007; Winter *et al.* 2007). The strong expression is maintained in the vasculature in 7-day-old seedlings, whereas only a weak GUS staining of the hypocotyl, epidermis and cortex is discernible (Turano *et al.* 2002). After 7 weeks, the GUS staining pattern remains unchanged but the expression intensity is much stronger.

AtGLR3.3

The AtGLR3.3 receptor shows an expression in guard and mesophyll cells of the leaf and it seems to be one of the few that is clearly expressed also at the root apex (Brady *et al.* 2007; Cho *et al.* 2009). However, the strongest AtGLR3.3 GUS staining can be seen in rosette leaves (Manzoor *et al.* 2013).

AtGLR3.4

Similar to other AtGLRs this gene is expressed in all tissues of the seedling and can be detected in protoplasts derived from mesophyll and guard cells (Meyerhoff *et al.* 2005). An AtGLR3.4-GUS line shows a high expression in cotyledons and young roots, whereas in adult plants the expression expands to mesophyll cells, vascular bundles and hydathodes of developing leaves (Meyerhoff *et al.* 2005). Expression in the root tissues (cortex, epidermis, root hairs) is weak compared with its strong expression in leaves and stems (Meyerhoff *et al.* 2005). Nevertheless, the most intense GUS staining was observed in rosette leaves (Manzoor *et al.* 2013). AtGLR3.4 is probably involved in processes connected to the early phloem (like AtGLR3.2) since its mRNA level is much higher in the protophloem than in the mature one (Brady *et al.* 2007; Vincill *et al.* 2013).

AtGLR3.5

The glutamate receptor AtGLR3.5 could be involved in early and late developmental processes. Its gene expression in germinating seeds is strongly upregulated but only during the actual germination course (Kong *et al.* 2015). Another expression peak is observable in a later developmental stage of 5-week-old plants (Teardo *et al.* 2015). In both cases, the onset of AtGLR3.5 gene expression is related either to seed germination events or to a regulation of senescence in leaves (Kong *et al.* 2015; Teardo *et al.* 2015).

Furthermore, AtGLR3.5-GUS lines show also staining of the shoot and root of young seedlings and an even stronger labelling of embryonic cotyledons (Kong *et al.* 2015).

AtGLR3.7

Investigations on a single cell level revealed that this receptor is the only one that is expressed in every cell (type) throughout the plant and its GUS expression in all tissues underlines that it has probably essential functions as a signal transmitter or ion transporter in *A. thaliana* (Roy *et al.* 2008). Its ubiquitous presence in plant cells could also indicate a fundamental role in the assembly of the glutamate receptor complex (see 'Molecular structure') in which it could serve as a platform for the other three glutamate receptors (Roy *et al.* 2008). Otherwise, the assumption of a clear phenotype due to a knockout of this receptor has not yet been reported and contradicts its hypothetical importance.

Taken together, GLRs seem to fulfil an ancestral function in *A. thaliana* since an expression of these receptors is confirmed in every plant cell/tissue (Fig. 3). Even if there is no consistent expression pattern on a cellular level detectable (Chiu *et al.* 2002; Roy *et al.* 2008), the presence of at least five members of these receptors in each single cell emphasises a central role in basic cellular processes. An explanation for the lack of stringency in respect to the expression levels could be a high degree of redundancy due to the gene duplication events. This redundancy can be seen also as an ubiquitous need for these receptors and it could offer a chance for a specialisation of distinct GLRs. A very good example represents AtGLR clade II in which a limitation of gene expression could take place due to a substitution by members of its sister clade I.

Origin of GLRs and their relation to glutamate receptors in other kingdoms

During the last few years, profound evidence for a common origin of animal iGluRs and plant GLRs was reported. It seems that an amino acid signalling based on glutamate receptors existed before the divergence of plants and animals. The prokaryotic glutamate receptor (GluR0) from the bacterium *Synechocystis* sp. represents an intriguing example. This receptor is capable of binding L-glutamate, glycine and L-serine but neither NMDA nor AMPA (Chen *et al.* 1999). GluR0 exhibits a homology of 24% with a glutamate receptor of cyanobacteria (GluRG3) and a similarity of 22% to *A. thaliana* glutamate receptor AtGLR3.4 (Teardo *et al.* 2011).

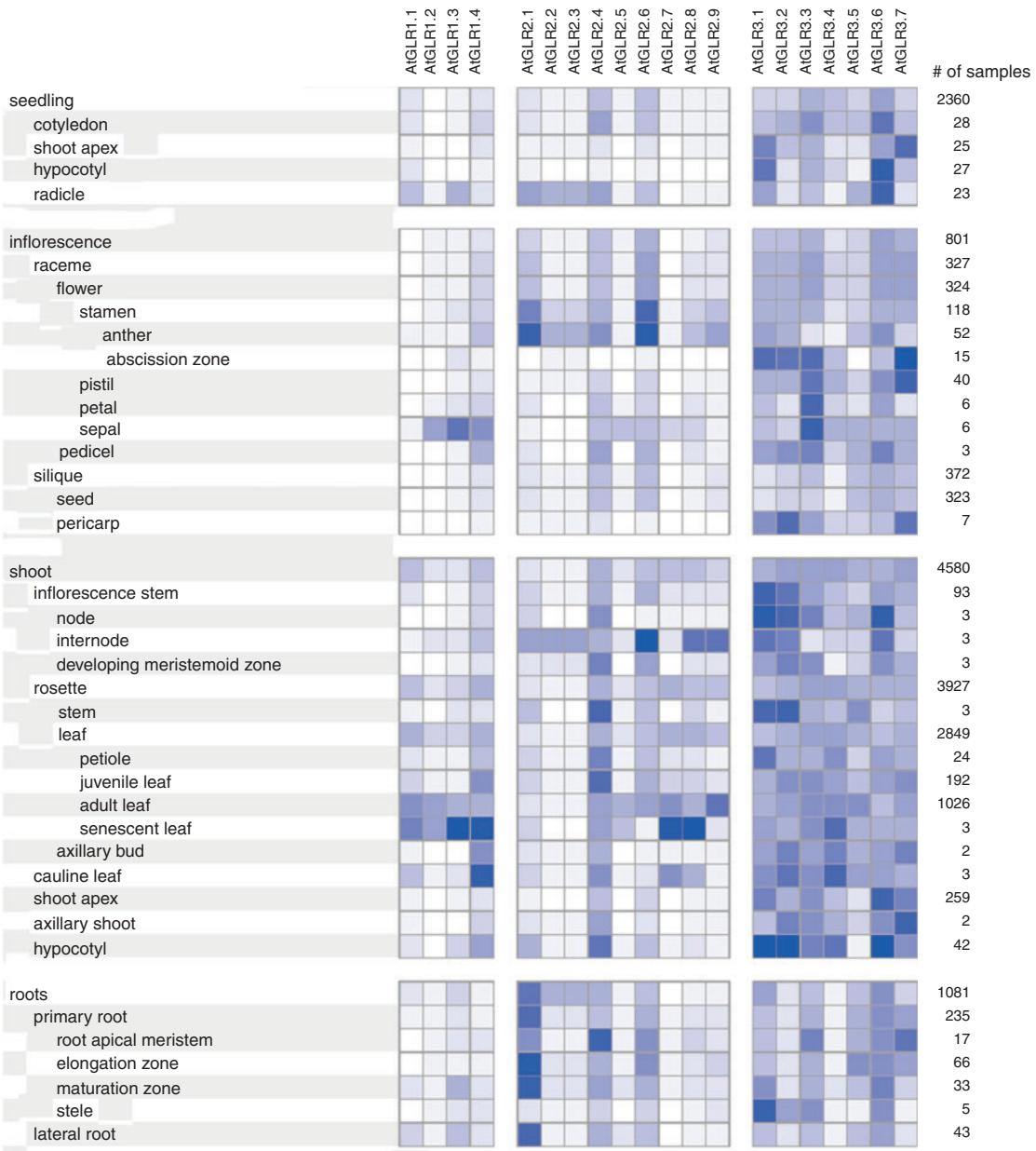

Fig. 3. Expression analysis of all 20 glutamate receptor-like receptors of *Arabidopsis thaliana* in different plant tissues and developmental stages. White: no expression, blue: high expression. Adapted data obtained from Genevestigator (Hruz *et al*. 2008).

All these glutamate receptors could originally have evolved from prokaryotic potassium channels, as it was proposed for animals first (Galen Wo and Oswald 1995) and later for plants (Demidchik *et al*. 2002). Further evolution of common ancestors of iGluRs in animals and GLRs in plants would have been accomplished even before the branching out of the today known animal iGluR varieties of kainate, AMPA and NMDA receptors (Chiu *et al*. 2002). This means an early assembly of the four transmembrane domains M1–M4 and the ligand binding sites S1 and S2 before the divergence of plants and animals (Chiu *et al*. 1999). Co-evolution of animal iGluRs and plant GLRs after the separation into plants and animals would support an essential role for these proteins and their necessary presence in these two kingdoms. Over such a long period of time, only minor variations between both GLRs and iGluRs occurred, which underlines also the importance of their way of working in these organisms.

The outcome of phylogenetic studies aiming in understanding the relationship of GLRs and iGluRs depends strongly on the tool/software and the chosen part of the amino acid sequences that were used for their analysis. In its first description of GLRs, Lam *et al*. (1998) emphasised one of the highest sequence similarity of GLRs with kainate/AMPA receptors (non-NMDA receptors). In accordance with this, a very close relation of GLRs to AMPA receptors was claimed by Nagata (2004), whereas Kim *et al*. (2001) argues for a link of a member of

AtGLRs (AtGLR3.2) to kainate receptors. Hereby, AtGLR3.2 could be an exception due to its minor sequence identity to iGluRs of only 21%, although there is a 61% identity within the M3 region (Kim *et al*. 2001). In contrast, there are authors who assume the closest relation of GLRs to NMDA receptors, especially to the NMDA subunits GluN1 and GluN3 (Chiu *et al*. 1999; Lacombe 2001; Dubos *et al*. 2003).

When comparing the amino acid sequences of AtGLRs with rat iGluRs, the strongest overall sequence identity seems to exist between AtGLRs and non-NMDA receptors with the highest similarity of AtGLR clade III to AMPA and kainate receptors (Table 2). However, the animal NMDA receptor subunit GluN1 displays the strongest similarity to AtGLRs, irrespective if taken all *Arabidopsis* GLRs together, or if AtGLR clades are compared with single iGluRs. Following this model, the clade III is the closest relatives to animal glutamate receptors.

Table 2. Theoretical distance of glutamate receptors of *Arabidopsis thaliana* and ionotropic glutamate receptors of *Rattus norvegicus*

Distance computation based on amino acids sequences of all 20 glutamate receptor-like receptors of *Arabidopis thaliana* and all 18 ionotropic glutamate receptors of *Rattus norvegicus*. A low number indicates a high degree of similarity. Pairwise arrangement in numeric order beginning with highest sequence identity. Abbreviations of iGluRs correspond to guidelines established on website available at http://www.guidetopharmacology.org/LGICNomenclature.jsp (accessed 15 May 2015). Distance calculated with MEGA6 software (Tamura *et al*. (2013)

AtGLR clade	iGluR	Distance	AtGLR (specific)	Distance to iGluRs (in total)
Clade III	GluN1	0.774	AtGLR 3.6	0.798
Clade III	GluK2	0.780	AtGLR 3.4	0.799
Clade III	GluK1	0.788	AtGLR 3.2	0.802
Clade III	GluD2	0.789	AtGLR 3.1	0.802
Clade III	GluD3	0.796	AtGLR 3.3	0.803
Clade III	GluK3	0.797	AtGLR 3.7	0.805
Clade III	GluK5	0.800	AtGLR 3.5	0.806
Clade III	GluA1	0.801	AtGLR 2.9	0.807
Clade III	GluK4	0.802	AtGLR 2.2	0.809
Clade III	GluA2	0.804	AtGLR 2.8	0.811
Clade II	GluD2	0.804	AtGLR 2.5	0.814
Clade II	GluK2	0.805	AtGLR 1.1	0.814
Clade III	GluA4	0.805	AtGLR 2.7	0.815
Clade II	GluK3	0.805	AtGLR 2.3	0.815
Clade II	GluN2c	0.806	AtGLR 2.6	0.815
Clade II	GluD1	0.807	AtGLR 2.1	0.816
Clade II	GluA1	0.808	AtGLR 2.4	0.819
Clade I	GluD2	0.808	AtGLR 1.2	0.820
Clade I	GluD1	0.810	AtGLR 1.4	0.822
Clade II	GluN1	0.810	AtGLR 1.3	0.822
Distance between subgroups				
Clade III	Delta	0.792	*Distance between AtGLR clades and iGluRs (in total)*	
Clade III	Kainate	0.793	Clade III	0.802
Clade III	AMPA	0.805	Clade II	0.813
Clade II	Delta	0.806	Clade I	0.820
Clade I	Delta	0.809	–	–
Clade II	Kainate	0.810	–	–
Clade III	NMDA	0.810	–	–
Clade II	AMPA	0.816	–	–
Clade II	NMDA	0.817	–	–
Clade I	Kainate	0.818	–	–
Clade I	NMDA	0.822	–	–
Clade I	AMPA	0.823	–	–

GLRs in other plant species

Most research is dealing with GLRs of *A. thaliana* since their discovery in 1998. As these receptors seem to be part of a fundamental signalling mechanism, an ever increasing number of publications are reporting the existence of glutamate receptors in other plant species. An overview about the phylogenetic relationship of plant GLRs and animal iGluRs is given in Fig. 4.

Already in the first studies concerning glutamate receptors in *Arabidopsis*, it was mentioned that AtGLR-related genes exist in other plants (Lam *et al*. 1998). However, it took 8 years until the first discovery of GLR-like proteins (RsGluR) in small radish *Raphanus sativus* L. (Kang *et al*. 2006). The RsGluRs contain all six conserved domains found in iGluRs and GLRs. Their sequence exhibits a strong identity to AtGLR3.2 (Kang *et al*. 2006). A hydrophobic signal peptide for the secretory pathway as well as an endoplasmic reticulum (ER) retention signal is located within the *C*-terminus. If mRNA splicing occurs at these receptors then there are two different localisations possible, one at the plasma membrane and another one within the ER. A localisation of one of these receptors within the plasma membrane could be verified by a transgenic RsGluR:GUS-mGFP *Arabidopsis* line (Kang *et al*. 2006). The other location within the ER membrane exists so far only in theory but GLRs could be involved in the regulation of Ca^{2+} fluxes originated from this important Ca^{2+} store.

Oryza sativa L. is another plant species in which GLRs are characterised. The rice genome encodes 24 different glutamate receptors (OsGLR) and they are very similar to AtGLRs of clade III (Li *et al*. 2006; Singh *et al*. 2014). They cluster into four groups while group I, II and III seem to have a common ancestor with *A. thaliana* (Singh *et al*. 2014). However, group IV is rice-specific and came probably into existence by gene duplications and a following mutation of one of these genes (Singh *et al*. 2014). All receptors found in rice contain the six conserved domains as well as all 37 invariant amino acids of the AtGLR gene family, supporting their relation to *Arabidopsis* or a common ancestor of both (Li *et al*. 2006). The M2 domain is very similar to that of a kainate receptor subunit but the pore region of rice GLRs contains an additional fifth transmembrane domain (Li *et al*. 2006; Singh *et al*. 2014). Its function is so far unknown, but it could change, improve or extent the ion selectivity of the formed receptor complex. Analogous to other GLRs also these glutamate receptors seem to enter the secretory pathway as shown for an OsGLR3.1-GFP fusion protein (Li *et al*. 2006). Furthermore, there is evidence that OsGLR3.1 is capable of forming homo-multimers in HEK cells. This is supporting the hypothesis of multimeric GLR complexes acting as the actually active receptors. The GFP-tagged protein and the homo-multimers were nonetheless retained within the ER (Li *et al*. 2006). A same retention within the ER was observed by Singh *et al*. (2014) when expressing a GFP-tagged version of OsGLR1.1 in tobacco leaf cells.

Other GLR-like genes found in tomato were named SlGLRs. Their protein domains have a similar structure as the ones of

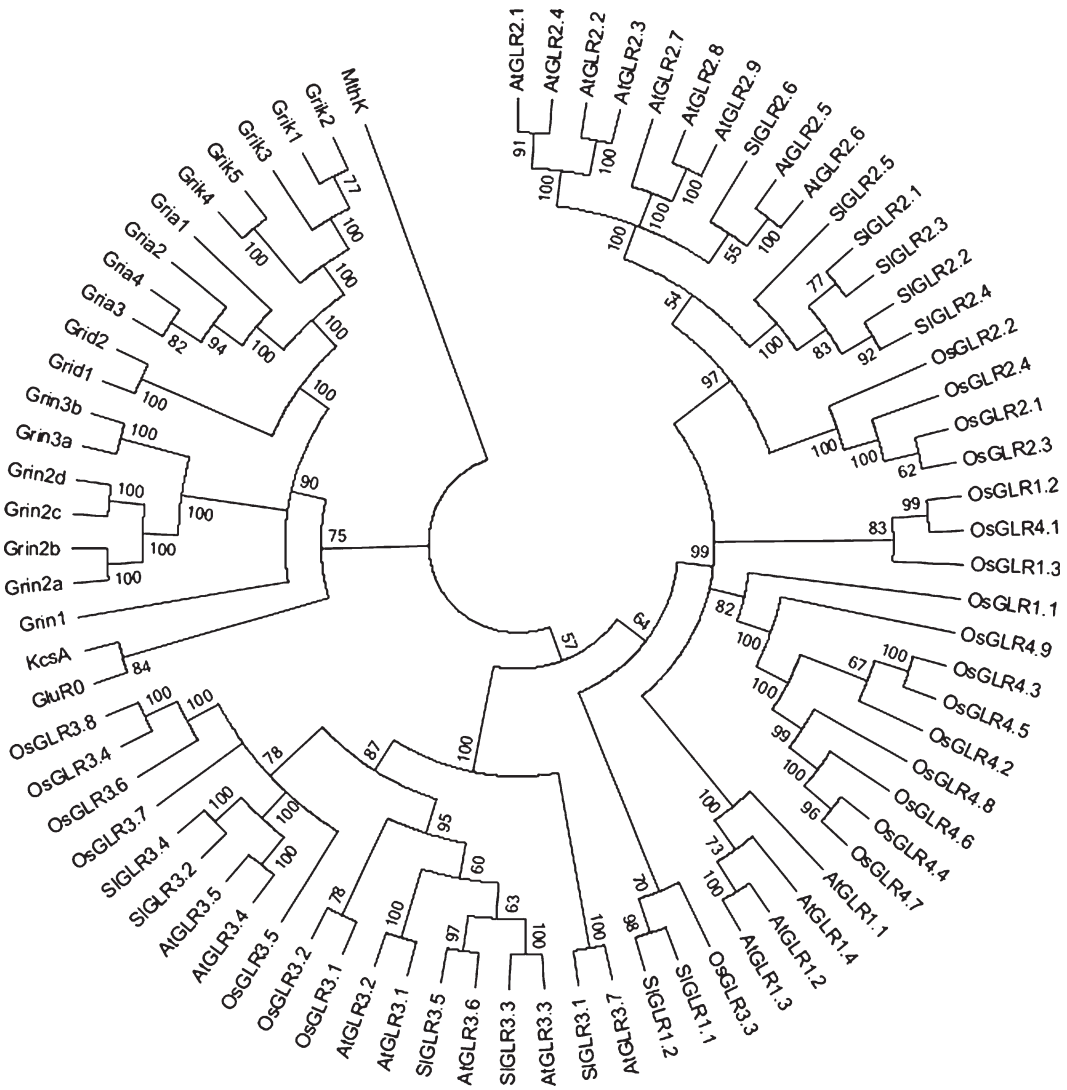

Fig. 4. Phylogenetic tree of glutamate receptors of three different kingdoms. Plant glutamate receptors are represented by all 20 AtGLRs of *Arabidopsis thaliana*, the 13 SlGLRs of tomato and the 24 OsGLRs of *Oryza sativa*. The animal glutamate receptors displayed are the 18 iGluRs of *Rattus norvegicus*. A glutamate receptor form the cyanobacterium *Synechocystis sp*. is represented as GluR0. Displayed are also the two prokaryotic potassium channels KscA from *Streptomyces lividans* and MthK from *Methanobacterium thermoautotrophicum*. Numbers indicate bootstrap values of 150 repetitions. Calculations were based on the respective amino acid sequences and were performed using MEGA6 software (Tamura *et al*. 2013). Amino acid sequences were obtained from UniProt (http://www.uniprot.org) for iGluRs and bacterial ion channels, the Rice Genome Annotaion Project (http://rice.plantbiology.msu.edu/) for OsGLRs as well as from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) for AtGLRs and SlGLRs. Accession date 20.05.2015.

AtGLRs and a signal sequence for the secretory pathway is contained within their *N*-terminal region (Aouini *et al*. 2012). The 13 SlGLR genes are subdivided into three clades whereas SlGLR clade II and III show a close relationship to AtGLRs (Aouini *et al*. 2012). Only clade I seems to be tomato-specific. These results imply a development of GLRs in an ancestor of land plants before divergence of *Solanaceae* and *Brassicaceae*, whereas AtGLR clade I was either lost during the evolution of the tomato or it was never incorporated into the genome of tomatoes from the beginning (Aouini *et al*. 2012). Indeed, only SlGLR2.6 has a close relation to AtGLR clade II. All the other members of this group show deviations within their amino acid sequence or are located on different chromosomes. It is possible that they came into existence by extensive gene duplication events as it is thought for other GLRs.

Proteins with corresponding structures to GLRs were also found in the wild grass *Echinochloa Crus-galli* (L.) Beauv. One gene similar to AtGLR clade III is present in the genome of this plant and was named 'EcGLR1' (Li *et al*. 2013*b*). Although its base pair and amino acid sequence show some likeness to common AtGLRs, there are deviations within the structure of the whole protein. The positions of the two ligand-binding

domains are located at opposing ends of the protein (Li *et al.* 2013*b*). One of them is situated at the *N*-terminus like in other GLRs but the other ligand-binding domain is found at the *C*-terminus (Li *et al.* 2013*b*) which accounts either for a modified function or a completely different protein.

The presence of GLR or GLR-like genes and proteins in all plant species analysed so far suggests that all plants or even all higher organisms carry genes that encode for these related receptors. Their central role within the kingdoms of life could be explained by a regulation of basic Ca^{2+} signatures linked to electric activities at the plasma membranes accomplished via their ion conducting properties.

Electrophysiology

One of the most elementary functions of animal iGluRs is the transduction of signals within the nervous system. This is achieved by a ligand-gated, rectified influx/efflux of mainly cations (Na^+, K^+, Ca^{2+}) across the plasma membrane of neurons. An activation of iGluRs by their respective agonists (e.g. NMDA, AMPA and kainate) causes membrane depolarisations and accompanied vesicle fusions to the plasma membrane. In this way, a release of further agonists/antagonists into the extracellular matrix (here: the synaptic cleft) leads to a transduction of the electrical signal from a cell to the next (Traynelis *et al.* 2010).

At the beginning of the discovery of GLRs in plants, it was not known whether these receptors are also capable of conducting ion fluxes similar to their animal homologues. The first studies were aimed in unravelling this important question. Soon it became clear that in plant cells, glutamate elicits in a dose-dependent manner an increase of cytoplasmic Ca^{2+} levels and plasma membrane depolarisations (Dennison and Spalding 2000). The observable currents/depolarisations are prevented by a treatment with Ca^{2+} chelators such as ethylene glycol tetraacetic acid (EGTA) or unspecific ion channel blockers like La^{3+} or Gd^{3+}. This strongly suggests the presence of glutamate-inducible calcium channels in membranes of plant cells (Dennison and Spalding 2000).

The amino acid glycine seems to be another main agonists besides glutamate since a treatment with this amino acid evokes a dose-dependent Ca^{2+} influx across the plasma membrane, too (Dubos *et al.* 2003). The iGluR antagonist DNQX is capable to prevent these glutamate- and glycine-induced Ca^{2+} fluxes – but only in aerial and not underground tissues (Dubos *et al.* 2003). This demonstrates not only an intense similarity between GLRs and iGluRs since DNQX is one of the most effective antagonist of iGluRs, but also the existence of different subsets of GLRs with their own specific sensitivities against agonists and antagonists (Dubos *et al.* 2003).

Further evidence for an involvement of AtGLRs in the described observations is given by a study of Teardo *et al.* (2010). Planar lipid bilayers, enriched with vesicles made from the inner chloroplast membrane of spinach, contain members of the AtGLR clade III and were shown to increase ion currents when treated with glutamate or glycine. This divalent cation-conducting activity is prevented by DNQX (Teardo *et al.* 2010). A heterologous expression of AtGLR3.4 in human embryonic kidney (HEK) cells causes Ca^{2+} inward currents that increase when glutamate or glycine is applied (Vincill *et al.* 2012). In addition, the amino acids asparagine, serine, alanine and phenylalanine are also capable of inducing this kind of currents. A control transfection with only an open reading frame for a fluorescent protein did not lead to a change of ion currents when treated with any amino acid. During this study the effective amino acid concentrations range between 0.01–10 mM (Vincill *et al.* 2012). GLRs seem to possess a high selectivity and a subtle sensitivity against various ligands since also other amino acids than glutamate and glycine exert similar effects on ion currents in a concentration dependent manner.

Glutamate exerts a noteworthy electrophysiological effect also in other plant species. In a study by Felle and Zimmermann (2007), an application of glutamate caused action potentials in a monocotyledon that were presumably elicited by a binding of the amino acid to a receptor/Ca^{2+} channel. The measured electrical signals do not represent a plain plasma membrane depolarisation but complex action potentials as can be concluded by different ion fluxes and the propagation of the action potentials from one leaf to another (Felle and Zimmermann 2007).

A second and rarely recognised study was conducted on the liverwort *Conocephalum conicum* (L.) Dum.: it revealed that even this phylogenetic quite distant organism is responsive to glutamate, glycine and NMDA, although at much higher concentrations (Krol *et al.* 2007). Glutamate and glycine at levels between 0.5 and 20 mM as well as 10 mM NMDA caused action potentials with a refractory period of 1 h whereas control treatments using the amino acid asparagine did not led to any similar phenomenon. The refractory period is similar to the desensitised state in *A. thaliana* (see 'GLR-mediated currents in detail') and could indicate a mechanism based on potential GLRs in liverwort.

Nevertheless, the observed effects showed also some distinct deviations. The amplitude of the action potentials is not affected by the amino acid concentration and a second treatment with the same amino acid does not cause another action potential. When the second treatment is done by a different amino acid (application of glutamate/glycine in an alternating order), however, two action potentials are detectable. The Ca^{2+} component of an action potential could be observed only in a treatment with 15 mM glutamate/glycine and is actually made of a glutamate-/glycine-induced hyperpolarisations or depolarisations. The first one could originate from a H^+-coupled transport or H^+-coupled forces such as H^+-ATPases at the plasma membrane while only the latter one involves true GLR-like activities. Related to this, alone the depolarisation induced by glutamate was prevented by DNQX. Together, this accounts for two separate phenomena that are leading to the hyper- and depolarisation (Krol *et al.* 2007).

In summary, the analogy between a liverwort and *Arabidopsis thaliana* is based on two observations. First, the effects of glutamate, glycine and NMDA are prevented by an application of AP-5 or La^{3+}, whereas DNQX prevents only the effect exerted by glutamate on the potential GLRs as well as the Ca^{2+} component of the action potentials in plants (Krol *et al.* 2007). Second, single applications of 0.1 mM glutamate or glycine do not lead to an action potential but the simultaneous application of both amino acids at the same concentrations causes one – a phenomenon

also observed for glycine and NMDA (Krol *et al*. 2007). This synergistic action of GLR agonists was already reported for *A. thaliana* earlier (Dubos *et al*. 2003).

GLRs appear to be more sensitive than expected. In the first study about the electrophysiological characteristics of AtGLRs, glutamate concentrations ranging from 0.3 mM to 3 mM with the strongest effect at 1 mM were established (Dennison and Spalding 2000). There are some indications accounting for lower effective concentrations of potential GLR ligands. Applications of 0.1 mM glutamate or another agonist evoke already a significant response, causing an GLR activation up to 40% (Vatsa *et al*. 2011; Vincill *et al*. 2012). Apparent changes in the root architecture are detectable in applications of ~0.05 mM L-glutamate (Walch-Liu *et al*. 2006) although even lower concentrations down to 0.01 mM were suggested by Dubos *et al*. (2003).

Furthermore, their actual responsiveness is determined not only by the concentration of one single agonist or antagonist but it is affected in a synergistic manner by different ligands at the same time. The intensity of $[Ca^{2+}]_{cyt}$ fluctuations caused by a treatment with 1 mM glutamate or glycine is the same as for a simultaneous application of both amino acids at a concentration of 0.01 mM (Dubos *et al*. 2003). We note that while treating the plants simultaneously, an increase of the amino acid concentrations above 0.01 mM does not further raise $[Ca^{2+}]_{cyt}$ (Dubos *et al*. 2003). This indicates a saturation of the receptor at very low concentrations, despite the fact that in general the K_m value of GLRs is calculated between 0.2 and 0.5 mM (Demidchik *et al*. 2004).

GLR-mediated currents in detail

Since plant GLRs are supposed to be close homologues to iGluRs, main characteristics of these receptors (and electrical signalling receptors in general) include a high sensitivity to agonists/antagonists and also the ability of desensitisation to avoid constitutive signalling. Similar to iGluRs, plant GLRs seem to be involved only in the initial steps of Ca^{2+} signalling by mediating the first transient Ca^{2+} influxes. These primary fluxes could be a trigger for the activation of other receptors/channels, which leads to the $[Ca^{2+}]_{cyt}$ elevations detected in electrophysiological studies. When looking closer on the ligand-evoked currents, it seems that the application of an agonist like L-glutamate does not cause a simple depolarisation of the corresponding membrane due to an efflux/influx of a single sort of ions. In contrast, the progression of a glutamate-induced Ca^{2+} influx features some distinct stages.

At the beginning of a GLR agonist treatment, a strong and rapid Ca^{2+} influx occurs, which leads to increases of $[Ca^{2+}]_{cyt}$ (Demidchik *et al*. 2004; Vatsa *et al*. 2011). The cytoplasmic Ca^{2+} elevations seem to originate from entering of extracellular Ca^{2+} into the cell (Vatsa *et al*. 2011). Here, the first inward currents arise within seconds and they are followed by channel-burst like events. During that time, glutamate-induced spiky components of the Ca^{2+} inward currents occur whereas the Ca^{2+} outward currents seem to be unaffected by the agonists treatment. Following the heavy influx of Ca^{2+} within the first 10 min, $[Ca^{2+}]_{cyt}$ decreases slowly within 1 h and leads to a new steady-state Ca^{2+} activity that is ~2 to 3 times higher than before. The maximal elevation of the Ca^{2+} is ~6–7 times higher and occurs at GLR agonist concentrations between 0.03 and 1 mM (Demidchik *et al*. 2004). In general, $[Ca^{2+}]_{cyt}$ increases constantly within an agonist concentration ranging from 0.03 to 5 mM but the first significant increase of currents is already observed at ~0.1 mM (Demidchik *et al*. 2004; Vatsa *et al*. 2011).

The actual plasma membrane depolarisation seems to be a dose- and threshold-dependent event. An application of 0.05 mM glutamate induces only weak and transient depolarisations of the membrane while a treatment with 0.5 mM causes a much stronger and longer-lasting depolarisation (Meyerhoff *et al*. 2005). In both cases, this is followed by a repolarisation that proceeds within 2 min and lasts until the resting potential is reached again. An increase of the agonist concentrations up to 5 mM does not cause a more intense or longer-lasting membrane depolarisation. However, similar to lower concentrations, a transient and dose-dependent increase of $[Ca^{2+}]_{cyt}$ peaks after a few seconds and fades within 20 s. The $[Ca^{2+}]_{cyt}$ changes are supposed to precede the actual plasma membrane depolarisation. This would indicate an activation of other channels such as Ca^{2+}-dependent ion channels after the initial activation of GLRs by agonists and the accompanied first Ca^{2+} influxes (Meyerhoff *et al*. 2005).

GLRs are likely to become desensitised upon sequential ligand applications. A second agonist treatment results in a reduced depolarisation amplitude and even 1 h after the initial treatment, the amplitude is still diminished up to 50% (Meyerhoff *et al*. 2005; Stephens *et al*. 2008). Also the agonist-induced membrane depolarisations are affected. The Ca^{2+} influx evoked by an application with a GLR agonist at a concentration of 1 mM is reduced up to 56% due to a pretreatment with the same agonist at lower concentrations ranging between 0.01 mM and 0.1 mM (Qi *et al*. 2006).

Whether the pH affects the desensitisation of GLRs is not known. Qi *et al*. (2006) demonstrated that a desensitisation was prevented at a more acid pH of 5.7 in root cells of *A. thaliana* whereas another study by Stephens *et al*. (2008) found no differences in the desensitisation behaviour of GLRs depending on changes of the pH in hypocotyls cells. Either are different subsets of pH-sensitive AtGLRs expressed in root and shoot or the effect of a H^+-coupled symport varies between these two parts of the plant. Nevertheless, the initial response to an agonist treatment does not differ at varying pHs (Qi *et al*. 2006; Tapken *et al*. 2013).

Some of these studies display the difficulties when investigating GLRs. It is possible that the often observed membrane depolarisations and Ca^{2+} influxes are caused by H^+-coupled symports of amino acids that occur at amino acid concentrations above 0.1 mM (Boorer *et al*. 1996). Even if the observed phenomena are only partly caused by this symport mechanism, further experiments should aim to exclude this possibility. For this reason, it is recommended to use lower agonist concentrations ~0.1 mM.

Another parameter should also be taken into account when observing glutamate-mediated Ca^{2+} changes. It could be shown that external Ca^{2+} influences the actual $[Ca^{2+}]_{cyt}$ rise. Thus, it could be demonstrated that a higher external Ca^{2+} concentration leads to an increased rise in $[Ca^{2+}]_{cyt}$ when pre-treated with 0.01 mM glutamate (Qi *et al*. 2006). The strength of the membrane depolarisation due to an application of 25 µM glutamate depends tightly on external Ca^{2+} concentrations

ranging from 1 to 10 mM (Qi *et al*. 2006). Both phenomena are the result of an enhanced Ca^{2+} influx due to large concentration gradients of Ca^{2+} across the plasma membrane, which are both tissue and development specific in plants.

In general, it is suggested to consistently incorporate the effects of antagonists and inhibitors within electrophysiological experiments to further support the hypothesis of amino acid-caused ion fluxes mediated by GLRs, which are missing in studies such as that by Vincill *et al*. (2012).

Physiological effects mediated by GLRs

The characterisation of plant glutamate receptors was hampered not only by difficulties during the cloning and expression process of the genes but also due to a probably high degree of redundancy among their members. This redundancy causes particular problems when trying to characterise single knockout lines. A double or triple knockout is much more likely to produce a clear phenotype. An alternative to that could be by using overexpression lines, although this is creating a highly artificial physiological environment. An impact on unrelated proteins and enzymes has to be taken into account because of a sheer strain of the transcription, translation or translocation machinery of the cell. Nevertheless, a lot of new findings have been obtained during the last 20 years supporting a widespread involvement of GLRs in plants.

Root architecture

The root system fulfils several specific tasks and its organisation often determines the function and capacities of the different species. Therefore, the root plays a pivotal role in plant growth and competitiveness. The plant's underground tissue seems to act as an important sensor to detect advantageous spots rich in organic nutrients, inorganic elements or water within the soil and it is involved in perceiving beneficial and harmful bacteria or fungi. Furthermore, the root is most often the only region in which an active and passive absorption of water, essential ions and other chemical compounds occurs. Although the detection of the plants own condition is essential for its functioning, a recognition of other plants is also of great importance. Therefore, many processes for differentiating between individuals of the same species and strangers are located within the root (e.g. Brenner *et al*. 2006; Baluška *et al*. 2009).

Glutamate is seen as one of the main agonists of AtGLRs and its effects in *Arabidopsis* roots were reported in several publications. Walch-Liu *et al*. (2006) investigated the exact impact of this amino acid on the plant root system at first in a broader study. Low concentrations of glutamate inhibit the growth of the primary root whereas lateral roots show an increased root branching. The inhibition of the root growth proceeds in a dose-dependent manner and varies between different *Arabidopsis* ecotypes (Walch-Liu *et al*. 2006).

The root apex is the most sensitive zone and glutamate-induced inhibition starts within the meristem where mitotic activity is ceased (Walch-Liu *et al*. 2006). Later on, glutamate exerts an impeding effect also on the cell expansion within the elongation zone. These impacts on the root tip are restricted to primary roots only, whereas lateral roots are insensitive until they reach a distinct length of 5–10 mm. The insensitivity could be explained by a developmental-determined expression of distinct glutamate receptors. A recovery of the root apex after a glutamate treatment depends on the initial glutamate concentration and the duration of exposure. After 4 days of permanent glutamate treatment, primary root apices are irrevocably damaged and are probably unable to recover due to a loss of meristem functionality (Walch-Liu *et al*. 2006) and damages to the transition zone (Sivaguru *et al*. 2003).

This kind of reorganisation of the root structure would enable plants to explore and exploit advantageous patches within the soil in a very efficient way. However, since the monitored effects could not be prevented by typical GLR antagonists like DNQX or AP-5, it is possible that either glutamate receptors of roots are insensitive to these agents or the observed effects are not strictly associated with GLRs.

Forde *et al*. (2013) explains the phenomena exerted by glutamate on the root system as a kinase-mediated event. Two agents, 2-(4-chloro-3-methylphenyl)-2-oxoethyl thiocyanate (CMOT) and 1-(2,6-dimethylphenyl)-2,5-dihydro-1H-pyrrole-2,5-dione (DDPD), prevent the inhibiting effect of glutamate on the primary root. These two chemicals were found to interfere with several kinases *in vivo*. Furthermore, a knockout line missing the kinase MEKK1 also lost its sensitivity to the glutamate-induced changes of the root architecture while being still sensitive to the effects of other amino acids like glycine, serine and cysteine (Forde *et al*. 2013). Therefore, MEKK1 seems to be involved in the downstream events of a glutamate signalling, which affects the root structure. We note that this specific kinase is involved in plant immune responses, too, and could act also in glutamate-induced and GLR-mediated defence processes (see 'Plant immunity').

The initiation of lateral root primordia seems to be regulated by the *Arabidopsis* glutamate receptors AtGLR3.2 and AtGLR3.4. A knockout of one of these two GLRs makes the transgenic plant lines to generate root primordia in a higher number (Vincill *et al*. 2013). The additional lateral root primordia are characterised by an early growth arrest followed by a complete inactivity. Notably, the whole root structure as well as the number of lateral roots is unaffected. This implies a co-ordination by GLRs of early phases in root development. The glutamate receptors could help to adjust a proper root branching in regulating cellular activities within the meristem and the transition zone.

Similar findings have already been made for GLRs within rice root apices (Li *et al*. 2006). A control of the organisation and function of the root meristem by a glutamate receptor was demonstrated in *O. sativa*. A knockout of OsGLR3.1 affects the whole root system including primary, lateral, as well as adventive roots and causes a shortening of the primary roots (Li *et al*. 2006). A disruption of the gene leads among others to an excessive apoptosis in the elongation zone which explains the shortened root. An OsGLR3.1 knockout causes severe damage to its tissue at the immediate root apex, too. A reduced root diameter is observable and can be explained by an inhibition of the radial expansion of cells in the transition zone. Nonetheless, the number of cell layers is increased in the lateral root cap. The quiescent centre and initial cells display a higher mitotic activity while the root tip in general shows a reduced mitotic

activity. Together with the observed premature differentiation of initial stem cells, this phenomenon generates aberrant cell divisions and cell developments within the root apex. The function of OsGLR3.1 could be developmental stage-dependent since this occurs only in younger plants until the second week after germination (Li *et al*. 2006).

Plant development

A notable feature of GLRs in plants is that they seem to be interchangeable between different species. There is evidence for an at least partly functioning of a glutamate receptor of small radish in the Brassicaceae *Arabidopsis thaliana*. An overexpression of this RsGLR leads to a plethora of developmental alterations in its host organism (Kang *et al*. 2006). The transgenic plant line is characterised by a dwarf phenotype with undeveloped lateral shoots, a changed inflorescence stage with abnormal flowers and leaves as well as a burst of localised cell deaths. Moreover, it exhibits a retarded development leading to a continuation of flowering even during the dispersion of seeds. An overexpression of AtGLR3.2, a related GLR from *Arabidopsis*, mimics this morphology (Kang *et al*. 2006) and points to a possibility in which both genes have not only an identical structure but are capable of being expressed and functional in different plant species.

The overexpression of RsGLR in *Arabidopsis* reveals an upregulation of stress-responsive genes, i.e. against bacterial infections, jasmonic acid-induced genes and touch-responsive genes (Kang *et al*. 2006). Furthermore, it could be demonstrated that this enhanced gene expression increases the resistance to necrotrophic fungi. An observed downregulation of abscisic acid (ABA)-mediated genes that are necessary for a controlled water stress response indicates another activity of RsGLR in ABA signalling (Kang *et al*. 2006). Further research is needed to determine if RsGLR and AtGLR3.2 are involved in ABA-mediated stomatal processes (see 'Abscisic acid signalling').

There is also evidence for an involvement of GLRs in plant reproduction. A look on the gene expression level shows four GLRs of *O. sativa* are upregulated while four others are downregulated when the plant enters the reproductive stage (Singh *et al*. 2014) and six AtGLRs are expressed alone in the pollen of *A. thaliana* (Pina *et al*. 2005). In respect to this, the knockout of either AtGLR1.2 or AtGLR3.7 of *Arabidopsis* causes a partial male sterility that is noticeable at a reduced number of seeds per silique (Michard *et al*. 2011). In this case, the reason seems to be a combination of a slowing growth and an abnormal morphology of the pollen tube. Both phenomena are caused by reduced Ca^{2+} oscillations at the tip of the pollen tube in spite of unchanged average calcium currents (Michard *et al*. 2011). The hypothesis that GLRs are involved in this phenotype is supported by the finding that a GLR antagonist (CNQX) leads to similar morphologies in wild-type plants (Michard *et al*. 2011). However, the amino acid D-serine could function as a possible AtGLR agonist in modelling Ca^{2+} oscillations responsible for pollen tube growth rate. An application of D-serine or glycine leads to a faster dose-dependent growth and a curly pollen tube morphology (Michard *et al*. 2011). Both amino acids can function as potential GLR agonists and they could activate Ca^{2+} channels at the tip of the pollen tube. This, in turn, could trigger higher Ca^{2+} influxes and would lead to negative currents, which shift the reversal potential to a more positive voltage (Michard *et al*. 2011). In addition, the frequency of Ca^{2+} oscillations increases, too, when GLR agonists are applied. D-serine seems to target a glutamate receptor clearly since all described effects could be (partly) inhibited or even reversed through the application of GLR antagonists. CNQX appears to be the most potent antagonist, in this respect, followed by DNQX and AP-5 (Michard *et al*. 2011).

Two facts support the idea for a D-serine-mediated GLR signalling in plant reproduction. D-serine can be found all over the pistil, especially strong at the ovule, and a knockout of the only serine racemase in *A. thaliana* exhibits the same pollen tube abnormalities that are evoked by GLR agonists and antagonists (Michard *et al*. 2011). Following the hypothesis of Michard *et al*. (2011), D-serine is released within the pistil, it binds at GLRs within the plasma membrane of the pollen tube and thereby helps navigating the pollen tube to the ovule.

Eventually, GLRs seems to be involved also in the last stage of the plant development. Plant senescence is another important process required for the development of new structures, or the disposal of no longer used plant parts. An AtGLR3.5 knockout line displays a premature senescence as well as a reduction of the chlorophyll content in leaves (Teardo *et al*. 2015). An involvement of this GLR is promoted by the fact that there is an upregulation of AtGLR3.5 gene expression in 5-week-old plants (Teardo *et al*. 2015). It will be necessary to determine the exact regulators that are responsible for a promoted gene expression in order to elucidate to which pathways GLRs are contributing.

Photo- and gravitropism

One of the first reported effects of an iGluR antagonist in plants is based on DNQX and its impairment of light signal transduction in *A. thaliana* (Lam *et al*. 1998). This antagonist of kainate and AMPA receptors strongly inhibits hypocotyl elongation even during light exposure and it reduces chlorophyll synthesis moderately (Lam *et al*. 1998). Interestingly, an application of glutamate and/or glycine counteracts these effects in a dose-dependent manner (Dubos *et al*. 2003). Both amino acids seem to exert a synergistic action since they are more effective when applied together in low concentrations than separately in a higher concentration. Also iGluRs are gated simultaneously by different agents, therefore AtGLRs have probably a similar gating mechanism and function.

Another light-dependent effect is caused by an application of the known iGluR antagonist BMAA. When plants are grown in the presence of this agent in light, their hypocotyl is elongated disproportionately and the angle in which cotyledons are separated is decreased (Brenner *et al*. 2000). This behaviour mimics the effect of DNQX and could mean that BMAA has an antagonistic activity in plants. The co-treatment with glutamate (or glutamine) is able to alleviate the actions of BMAA, which indicates the same target for both substances (Brenner *et al*. 2000). We note that when treated plants are grown in dark, the hypocotyl as well as the root are shortened which cannot be explained with a plain antagonism on GLRs. So

even if BMAA competes with glutamate at the same glutamate receptor, a special modification of the receptor must take place to allow a differentiated response to both chemicals.

Another important study was employing computer analysis to detect very small deviations between different plant lines. During this analysis, Miller *et al*. (2010) found impairments in root gravitropism in an AtGLR3.3 knockout line. The transgenic plant line responds to a reorientation of the root in respect to the gravitational stimulus between 5 and 10 h later compared with wild-type plants. Its response is less stable and more variable that points to a role for AtGLR3.3 in fine-tuning or adjusting gravitropism in *A. thaliana* (Miller *et al*. 2010).

Calcium homeostasis

Glutamate receptors in general are considered as Ca^{2+} channels. The first experimental proof was provided by Kim *et al*. (2001) with an AtGLR3.2 overexpression line. An enhanced gene expression of this GLR causes severe calcium deficiency symptoms such as browning of the whole plant, necrosis in growing points at leaf tips, reduced growth, curling and deformation of leaves or disintegration of petioles (Kim *et al*. 2001). In that study, the Ca^{2+} content was not affected within the plant (Kim *et al*. 2001). This would mean that either the distribution of Ca^{2+} does not work properly or the utilisation of external Ca^{2+} is impaired, which both make the overexpression line accumulating more Ca^{2+} in distinct parts of the plant than the wild type. One explanation is that the increased number of GLRs leads to serious disturbances in calcium transport throughout the plant. Actually, an exogenous application of additional calcium was able to abolish the deficiency symptoms (Kim *et al*. 2001). In this way, a supply of extra calcium in the soil would increase also the Ca^{2+} content inside the plant and as a consequence counteract the distribution problems. A hypersensitivity to Na^{+} and K^{+} but not Mg^{2+} or mannitol underlines this interpretation. The excessive presence of AtGLR3.2 within the plasma membrane could facilitate the uptake of cations from the soil (Kim *et al*. 2001). On the other hand, an anti-AtGLR1.1 line displays a high sensitivity to Ca^{2+} as well. In this case it leads to a strong root growth inhibition when the transgenic plants encounter elevated levels of Ca^{2+} (Kang and Turano 2003).

Both contradicting observations can be explained with the assumption that AtGLR1.1 represents a different class of GLRs that is involved in the perception of Ca^{2+} levels within the soil. It is known that specialised Ca^{2+} sensors such as CBL1 detect calcium within the rhizosphere (see Cheong *et al*. 2003), but a transduction of the Ca^{2+} status is likely to involve Ca^{2+} signatures. AtGLR1.1 could be a relevant mediator of these signals and a knockout of the respective gene would impair a proper plant reaction to external amounts of Ca^{2+}. An overexpression of this glutamate receptor could help to validate this assertion and examine if a susceptibility to salt stress is a common feature of GLR overexpression lines.

Plant stress signalling

Experiments on plant stress adaptation demonstrate a fast response to these stimuli in form of an altered gene expression of several AtGLRs (Meyerhoff *et al*. 2005). Touch, cold, wound or osmotic stress increases the amount of mRNA transcripts of AtGLR3.4 up to six times in *Arabidopsis* seedlings (Meyerhoff *et al*. 2005). Also a cytoplasmic acidification due to high amounts of amino acids or acid agents (i.e. GABA, malate, potassium acetate) causes a rise in AtGLR3.4 gene expression (Meyerhoff *et al*. 2005).

Ca^{2+} channels seem to be mediators of this response since an application of unspecific cation channel blockers like La^{3+} prevents the elevated mRNA transcript levels (Meyerhoff *et al*. 2005). This finding points to a regulation of GLR gene expressions by Ca^{2+} signatures when plants are undergoing stress reactions. Similar to the described response in *A. thaliana*, seven GLR homologues found in rice (OsGLRs) display an altered gene expression under abiotic stresses such as salt, cold or drought stress (Singh *et al*. 2014).

Drought tolerance is also an important feature of plants since most of them encounter a lack of water regularly. The consequences for the plants are severe considering the sequential osmotic and ionic disturbances within the organism that, among others, lead to a loss of protein function (see Wang *et al*. 2003). GLRs as potential Ca^{2+} channels are expected to fulfil a pivotal role in the mediation of drought stress signals. An overexpression of the two glutamate receptors OsGLR1 and OsGLR2 of *O. sativa* was found to increase drought tolerance not only in rice but also when these receptors are heterogeneously expressed in *Arabidopsis* (Lu *et al*. 2014). This report highlights the existence of a fundamental mechanism for drought tolerance where proteins with a highly conserved structure and function are involved since it demonstrates a cross-validation between monocot and dicot plants against a specific stress stimulus.

Sensing toxic environments is another crucial issue. Since plants are in general sessile organism, they have developed different sophisticated mechanisms to perceive harmful spots swiftly when exploring the soil. Aluminium cations possess a high toxicity for them because Al^{3+} forms various complexes with essential metabolic molecules such as enzymes, other proteins or second messengers (see Delhaise and Ryan 1995).

A typical response when encountering Al^{3+} is the inhibition of root growth and on a cellular level the depolarisation of the plasma membrane as well as microtubuli depolymerisations (Sivaguru *et al*. 2003). Joint applications of Al^{3+} and glutamate cause such effects much stronger and faster, whereas the addition of the AtGLR antagonist AP-5 or the unspecific cation blocker Ga^{3+} prevents the impact of Al^{3+} completely (Sivaguru *et al*. 2003). The chloride channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) impairs functioning of aluminium-gated anion channels involved in the response to Al^{3+} toxicity. However, an application of NPPB eliminates only the above described responses to Al^{3+} whereas a treatment with glutamate still evokes a plasma membrane depolarisation and a degradation of microtubuli (Sivaguru *et al*. 2003). It seems that aluminium cations cause an efflux of glutamate from the root, which, in turn, activates Ca^{2+} channels (likely AtGLRs) at the plasma membrane (Sivaguru *et al*. 2003). Only the subsequent calcium signalling evoked by glutamate leads to the actual plant response against Al^{3+}. In this case, glutamate would function as a kind of second messenger that is detected by GLRs. Glutamate receptors would convert this signal into a Ca^{2+} signature entailing further downstream events and function in this way as mediators for Al^{3+} toxicity reactions.

Abscisic acid signalling

Abscisic acid is another main actor in plants. It plays a pivotal role in different kinds of stress signalling as well as in pathways associated with seed dormancy and leaf abscission (Cutler *et al.* 2010). It seems that members of the *A. thaliana* glutamate receptors are part of a mechanism that enables the proper functioning of this plant hormone by influencing diverse abscisic acid signalling pathways involved in root development, seed germination and stomatal closure.

An anti-AtGLR1.1 line displays strongly raised ABA levels compared with control plants (Kang and Turano 2003; Kang *et al.* 2004). The ABA content, which was increased up to eight times, arises from elevated amounts of transcripts of stress-inducible enzymes/proteins known as important ABA biosynthesis regulators in a combination with a reduction of mRNA levels for proteins involved in a desensitisation against ABA (Kang and Turano 2003; Kang *et al.* 2004). The resulting accumulation of ABA within the plant causes an enhanced drought tolerance due to ABA's effects on stomata (Kang *et al.* 2004). The intensified presence of ABA1, 2, and 3 within guard cells leads to a constriction of stomatal opening and thereby causing water retention within the leaf (Kang *et al.* 2004). It seems reasonable that a reduced number of glutamate receptors causes a higher susceptibility to ABA-mediated stress signalling. The same anti-AtGLR1.1 line responds furthermore to an exogenous application of ABA extensively and it displays reduced seed germination as well as seedling retardations and its roots are growing much slower (Kang *et al.* 2004). A similar observation was made when expressing a glutamate receptor of small radish (RsGLR) in *Arabidopsis*. The downregulation of ABA-mediated genes that are responsible for a controlled water stress response in the Arabidopsis RsGLR overexpression line indicates also for this protein an involvement in ABA stress signalling (Kang *et al.* 2006).

GLR antagonists do also influence ABA levels in the transgenic lines. A treatment with DNQX increases the already augmented ABA levels even further whereas this agent has no effect on wild type plants (Kang and Turano 2003). By doing so, the heighted ABA content furthermore prevents seeds from germinating (Kang *et al.* 2004). We note that the effect of DNQX on the anti-AtGLR1.1 line can be alleviated by a simultaneous treatment with glutamate or BMAA. All three agents could have an impact on the same target, which, in this case, would be a ligand-binding site of an AtGLR (Kang and Turano 2003).

Recently, it was reported that also the repression of AtGLR3.5 significantly delays seed germination in *Arabidopsis* as well as it enhances ABA sensitivity of seedlings whereas the respective overexpression line displays the opposite effects (Kong *et al.* 2015). It seems that regulators for ABA-mediated developmental progressions are disturbed in the transgenic AtGLR3.5 plants because of misleading Ca^{2+} signals in form of reduced Ca^{2+}-induced Ca^{2+} increase (Kong *et al.* 2015). In accordance with this, the knockout of AtGLR3.5 leads to fewer transcripts of Ca^{2+} sensing proteins (Kong *et al.* 2015). Only an external application of supplementary Ca^{2+} is able to countermand the inhibition of seed germination by re-suppressing ABA signals and genes for ABA biosynthesis (Kong *et al.* 2015). Taken together this accounts for the necessity of AtGLR3.5 as a mediator of Ca^{2+} signals encoding a proper regulation of an ABA-mediated seed germination process.

Carbon metabolism

The assimilation of carbon dioxide and the assembly of carbohydrates are significant characteristics of plants. Different ways of binding CO_2 derived from the atmosphere have been developed and the subsequent formation of mono- and polysaccharides serves various purposes. Besides acting as an energy source, they are also imperative contributors to the structural constitution, for example in form of cellulose and hemicellulose. A regulation of carbon assimilation and utilisation is controlled by diverse carbon sensors on a cellular level. These sensors perceive endogenous carbon metabolites and mediate an activation or inhibition of the metabolic machinery producing or consuming carbohydrates (Coruzzi and Zhou 2001).

There are indications that glutamate receptors are involved in the perception of the C : N ratios in plants via sensing sucrose contents. A knockout of AtGLR1.1 leads to an inability to germinate on growth medium containing only a low amount of sucrose as the only carbon source (Kang and Turano 2003). Lowering the C : N ratio by supplying additionally NO^{3-} restores a germination of the mutant line. Both phenomena indicate an inhibiting effect of high C : N ratios on AtGLR1.1-impaired plants (Kang and Turano 2003). Supporting findings on a molecular level show that proteins and enzymes associated with the carbon and nitrogen metabolism are reduced in number or display a decreased activity (Kang and Turano 2003). However, only proteins and their isoforms apart from mitochondria and chloroplasts are affected.

Following the authors, a diminished amount of AtGLR1.1 causes a higher sensitivity to sucrose and therefore gives a false estimation about the available sugar. This in turn would lead to a GLR-mediated defective regulation of seed dormancy. A theory supported by elevated ABA concentrations within the anti-AtGLR1.1 seeds (see Kang and Turano 2003).

A mechanism in which the actual C : N status is communicated via GLR-mediated Ca^{2+} signals within the vascular tissue seems to be possible since two AtGLRs (AtGLR3.2 and AtGLR3.4) are concentrated at sieve plates within the phloem (see Vincill *et al.* 2012, 2013). This tissue would enable even a long-distance communication between distal parts of the plant and inform about their actual carbon and nitrogen status.

Stomatal movements and photosynthesis

A regulation of stomatal closure and opening is essential for photosynthetic processes and plays also a role in plant defence. A closing prevents unnecessary evaporation of water and it is crucial for adaptation to drought stress. During a pathogen attack, it prevents the penetration of microorganisms into the plant. In contrast, gas exchange and temperature control within the leaves is only possible when stomata are opened. Since photosynthesis is a main feature of plants a strict control of the associated aperture is mandatory (e.g. Jia and Zhang 2008).

The *A. thaliana* glutamate receptor AtGLR3.1 is expressed in guard cells of stomata abundantly, indicating an activity in this cell type (Cho *et al.* 2009). Indeed, the movement of guard cells

appears to be partly regulated by GLR-mediated Ca^{2+}-induced Ca^{2+} oscillations. An overexpression of AtGLR3.1 leads to strong deficits in regulating long-term stomatal closure (Cho *et al*. 2009). So far, two ways of programmed stomata closing have been described in plants: a short-term closure that is achieved by a sudden Ca^{2+} increase and a plasma membrane depolarisation; and a long-term closing that is regulated by specific Ca^{2+} oscillations (Allen *et al*. 2001). Only long-term closure is affected in the AtGLR3.1 overexpression line pointing to a disturbance of responsible Ca^{2+} signatures (Cho *et al*. 2009). The ion channels of the transgenic line respond to external applications of Ca^{2+} normally and causing an usually short-term closure (Cho *et al*. 2009). However, investigations of endogenous Ca^{2+} oscillations caused by exogenous Ca^{2+} show peculiar deviations compared with the wild type. This assumes that an impairment of these subtle Ca^{2+} fluxes in the AtGLR3.1 overexpression line causes defects in long-term control of stomata (Cho *et al*. 2009).

Aside from this, other glutamate receptors could be involved in photosynthetic processes directly. Antagonists of GLRs are found to hamper the production of oxygen in spinach (Teardo *et al*. 2010). A treatment with CNQX reduces the oxygen formation in chloroplasts but not in thylakoids. AtGLR3.4 seems to be a good candidate as a regulator of photosynthesis since a knockout of this receptor causes a reduction in the photosynthetic yield of PSII as well as a decreased capacity of non-photochemical quenching (Teardo *et al*. 2011).

Plant immunity

Immune defence holds a fundamental role in the survival of plants. Several key players are involved in this system that is triggered by the perception of pathogens such as fungi, bacteria and viruses. The recognition of these pathogens is often achieved through binding of distinct molecules originated from the pathogen itself or from concomitant substances and by-products. The respective receptors are mainly situated within the plasma membrane but can be found also within the cytosol of plant cells. An identification of so-called microbe-associated molecular patterns (MAMPs) usually follows a cation influx containing particular Ca^{2+} signatures. These Ca^{2+} signals are decoded and will lead to an activation/deactivation of enzymes mediated by kinases and phosphatases (Jones and Dangl 2006).

The application of distinct amino acids before a MAMP treatment desensitises ion channels that are associated with MAMP-induced responses (Kwaaitaal *et al*. 2011). Most of the used amino acids do not show any effects but a treatment with glutamate or asparagine reduces the Ca^{2+} influx caused by MAMPs in a dose-dependent manner (Kwaaitaal *et al*. 2011). Moreover, glutamate alone activates several mitogen-activated protein kinases (MAPKs) swiftly and that can be prevented by the GLR antagonist CNQX (Kwaaitaal *et al*. 2011). Similarly, an activation of MAPK signalling cascades provoked by a chitin treatment is impeded in the presence of glutamate, kynurenic acid or CNQX (Kwaaitaal *et al*. 2011). Since these kinds of cascades are regulated by a correct Ca^{2+} influx, an involvement of GLRs in the observed phenomena is highly likely. Usually, the presence of MAMPs like flg22, elf18 or chitin causes an upregulation of defence genes to perform the actual immune responses. In the study by Kwaaitaal *et al*. (2011) could be shown that a treatment with glutamate or kynurenic acid causes a strong reduction of some defence gene transcripts. The potential agonists of GLRs seem to counteract the MAMP-induced activations of calcium channels and therefore are probably involved in the formation of responses in an early stage of plant immunity.

Another study showed that glutamate activates the production of nitrogen oxide (Vatsa *et al*. 2011). An involvement of GLRs seems likely since antagonists of glutamate receptors (AP-5, DNQX and MK-801) counteract this effect of glutamate (Vatsa *et al*. 2011). Still, there is some uncertainty about the actual target or pathway because the evolution of nitric oxide (NO) is not followed by other typical defence responses like reactive oxygen species (ROS) production, MAPK activation or hypersensitivity responses (Vatsa *et al*. 2011). There is no clear evidence that glutamate alone induces a plant defence reaction even if a generation of nitrogen oxide ensures an application of this amino acid. However, considering the plethora involvements of glutamate, it is obvious that only a combination of different chemicals, ions and/or other stimuli will be sufficient to elicit such a specific reaction as a defence response.

Glutamate could furthermore function as a second messenger for MAMP-induced downstream events. The MAMP cryptogein causes generic plant immune responses like a rapid Ca^{2+} influx and it leads to downstream modifications on protein, RNA and gene levels (Vatsa *et al*. 2011). Both glutamate and cryptogein cause a fast $[Ca^{2+}]_{cyt}$ increase but cryptogein-treated cells are no longer responsive to a subsequent glutamate treatment (Vatsa *et al*. 2011). The responsible Ca^{2+} influx-mediating channels obviously lost their sensitivity. A further investigation in cryptogein-induced changes revealed an increase of glutamate in the apoplast in tobacco cultured cells (Vatsa *et al*. 2011). An efflux of glutamate could in turn lead to an activation of Ca^{2+} channels in the plasma membrane. The idea of an exocytosis-driven glutamate efflux is supported by the finding that BFA or cytochalasin, well known for their disruption of exocytosis, reduces the cryptogein-induced glutamate rise within the apoplast (Vatsa *et al*. 2011). Both inhibitors furthermore reduce the cryptogein-induced Ca^{2+} influx as well as the $[Ca^{2+}]_{cyt}$ variations at the beginning of the immune response (Vatsa *et al*. 2011). In this case, glutamate is involved downstream of the initial cryptogein-stimulated reactions (Vatsa *et al*. 2011).

It would be useful to discover the exact mechanism by which glutamate is secreted into the apoplast. The possibility of a vesicle-mediated delivery would entail a kind of glutamate storage within membrane-enclosed compartments inside the cell. Since this would resemble the situation in animals where glutamate is stored in synaptic vesicles in neurons, it is surely worthy to investigate the localisation of glutamate within cells, i.e. in using anti-glutamate antibodies.

GLRs are presumably also involved in a transduction of defence stimuli within the plant body. Three antagonists of GLRs (DNQX, CNQX and MK-801) are found to reduce oligogalacturonide-induced Ca^{2+} variations that are connected to common plant defence responses significantly (Manzoor *et al*. 2013). Besides a decrease of Ca^{2+} variations up to 65%,

an application of these antagonists leads to a lowered H_2O_2 production as well as nitrogen oxide evolution and it diminishes the upregulation of plant defence genes (Manzoor *et al*. 2013). Consequently, plants treated with one of these agents are impaired in coping with pathogens like biotrophic *Hyaloperonospora arabidopsidis* and necrotrophic *Botrytis cinerea* (Manzoor *et al*. 2013).

In this study, DNQX exerted the strongest inhibition, which argues for an important role of the respective mediating receptors.

A look on the AtGLR genes expression level reveals a downregulation of clade III GLRs under biotic stresses or the exposure of elicitors of plant defence signalling such as oligogalacturonides or flg22 (Manzoor *et al*. 2013). Members of clade III could actually function as main actors in the mediation of defence signals. All knockout lines of clade III display a higher susceptibility to the pathogen *B. cinerea* and most of them seem to be involved in the transmission of wounding signals (Manzoor *et al*. 2013; Mousavi *et al*. 2013). The knockout of AtGLR3.1, 3.2, 3.3 or AtGLR3.6 reduces wounding-evoked membrane depolarisations as well as the expression of defence gene JAZ10 (Mousavi *et al*. 2013). A double knockout of AtGLR3.3 and AtGLR3.6 is even more severe. A loss of both glutamate receptors impairs the transmission of wounding signals from damaged leaves to neighbouring leaves strongly (Mousavi *et al*. 2013). The percussion seem to be seriously since even exogenous-applied electrical stimuli cannot restore normal plant responses such as plasma membrane depolarisation or the upregulation of stress genes (Mousavi *et al*. 2013). In this respect, presumably AtGLR3.3 acts as a hub in transmitting wounding signals. Its knockout evokes a weakened immune response against the above-mentioned pathogen *H. arabidopsidis* due to a reduced production of NO and ROS as well as an impeded activations of defence genes in plants that are exposed to oligogalacturonides (Manzoor *et al*. 2013).

In addition, the double-knockout line displays a strongly altered gene expression in general when glutathione or cysteine as potential GLR agonists are applied (Li *et al*. 2013*a*). About 72% of the glutathione-controlled genes are AtGLR3.3-dependent and most of the genes/proteins are involved in plant defence signalling (Li *et al*. 2013*a*). AtGLR3.3 appears to be also here a key player in an even broader system of glutamate signalling since ~70 different glutamate-induced gene expressions are regulated by this receptor (Li *et al*. 2013*a*).

Comparison with ionotropic glutamate receptors in animals

Glutamate receptors in animals can be divided in the two different groups of mGluR and iGluR. The first group comprises integral membrane proteins with a G-protein–coupled activity whereas members of the latter group form actual ligand-gated ion channels within membranes. Such ligands as glutamate or glycine activate all these receptors and evoke enzymatic actions and ion fluxes mediated by mGluRs and iGluRs, respectively (Dingledine *et al*. 1999). The sequence and structure of GLRs in plants is strongly related to iGluRs of animals and therefore they are generally considered as their homologues. These both receptor classes seem to have a common ancestor that underlines their likeness (see 'Origin of GLRs and their relation to glutamate receptors in other kingdoms').

Ionotropic glutamate receptors are widely expressed within the central nervous system of animals where they mediate fast excitatory synaptic transmissions. Their involvements in neuronal functions range from learning and memory to long-term potentiation as well as synaptic plasticity (Maren and Baudry 1995). Since iGluRs participate in such relevant neurological activities, causes for neurodegenerative disorders like multiple sclerosis or Alzheimer's and Parkinson's disease originate partly in defective glutamate receptor functioning (Ikonomidou and Turski 1996; Ribeiro *et al*. 2010). A disproportionate stimulation of these receptors leads to excessive Ca^{2+} influxes into the cell. In this case, an over-activation of enzymes such as proteases and caspases occurs, which often results in apoptosis (Rameau *et al*. 2000). Recently, also implications in cellular transformation and cancer progression due to glutamate receptor malfunctions have been reported (Prickett and Samuels 2012).

The neurologic activity of iGluRs is based on a direct mediation of ion fluxes through the ion channel domain in mainly synaptic membranes. These fluxes of Na^+, K^+ and Ca^{2+} are gated by the receptor itself due to a binding of their respective agonists and antagonists. In this respect, iGluRs can be further divided into the four subgroups of NMDA, AMPA, kainate and two orphan receptors (GluD1, GluD2) grounded on their structure and ligands. NMDA receptors hold an exceptional role among these classes (Dingledine *et al*. 1999). Members of this subgroup are the only ones allowing the entry of a substantial amount of Ca^{2+} in addition to the monovalent cations Na^+ and K^+. As a consequence, excitatory postsynaptic potentials are able to increase $[Ca^{2+}]_{cyt}$, which can evoke intracellular signalling cascades due to a function of Ca^{2+} as a second messenger. Furthermore, NMDA receptors are the only voltage-dependent iGluRs since they are blocked by extracellular Mg^{2+} in a hyperpolarised state. This feature restricts a Ca^{2+} influx only to a depolarised plasma membrane. In this way, a temporal and/or spatial combination of different iGluR activations is required for more complex cellular responses. Another unique characteristic of NMDA receptors is their co-activation by two different ligands – glutamate plus either glycine or D-serine (see Dingledine *et al*. 1999).

As already mentioned in several parts of this review, the very high structural homology between GLRs and NMDA receptors could indicate similar gating and conducting properties as well as possible overlaps in functions. Both NMDA receptors and GLRs are supposed to consist of four subunits assembled as a hetero/-homotetramer and seem to be activated/deactivated by identical agonists and antagonists. Most of all, they conduct Ca^{2+} fluxes and therefore are involved in fundamental calcium signalling processes within cells. Nonetheless, it is to expect that new findings about GLRs will highlight as well major differences. The physiological organisation of a plant organism is too different from an animal. Plant tissues are more scattered within the plant body for obvious biological reasons. A loss of some parts like leaves or branches must not impair the plants viability

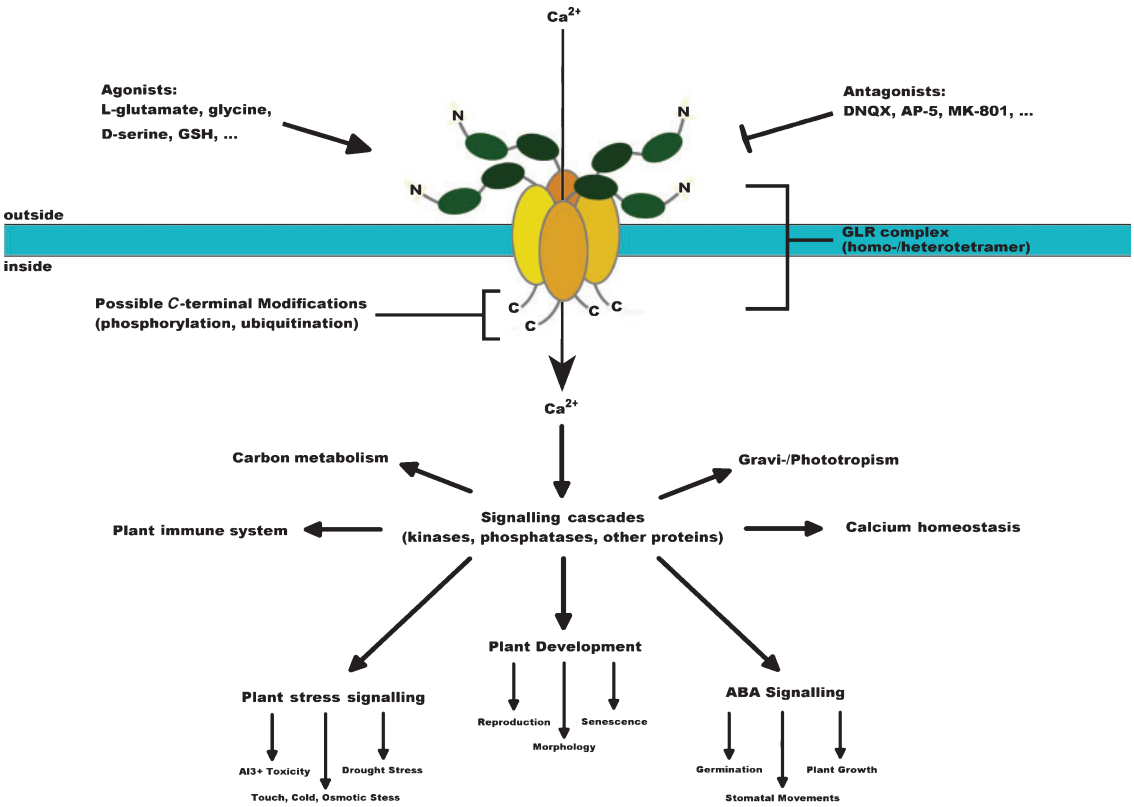

Fig. 5. Schematic overview of a glutamate receptor complex in *Arabidopsis thaliana* containing four GLR subunits. The displayed integral membrane proteins consisting of C-terminal domain, transmembrane domain (yellow), ligand binding sites (green) and N-terminal domain are capable of conducting mainly Ca^{2+} fluxes across membranes (blue) and causing Ca^{2+} signatures that are involved in various physiological processes.

in total. Furthermore, these plant compartments have to be reproducible in a fast and straightforward manner. In contrast, molecular mechanisms on a cellular level are quite conserved and they offer a possibility to customise an already established way of working in a new environment. More similarities and differences between GLRs and iGluRs are to be expected as we learn more about glutamate receptors in plants, but so far, iGluRs provides a reliable guideline to plan further investigations of GLRs.

Conclusions

During the last 15 years, considerable progress has been made towards a better understanding of GLRs in plants. Since these proteins are similar to their homologues in other species, it is reasonable to presume the existence of related amino acid signalling pathways within all main kingdoms of life. Depending on their occurrence, they seem to be essential for numerous cellular processes. Still, conserved structures of these calcium flux-mediating channels are a premise for their fundamental functions even today. Their exact way of functioning may vary between plants, animals and bacteria but prerequisites comprise a pore-forming domain incorporated into a membrane as well as ligand binding sites for regulating the passage of ions through the channel.

The involvements of GLRs in plants are manifold and the current knowledge argues for them as an indispensable element in plant signal transduction (Fig. 5). Nevertheless, further research will be necessary to provide deeper insights into their modulations on a molecular level and the complexity of interactions in which GLRs are the key players. Plant glutamate receptors include several glycosylation sites in their *N*-terminus and they provide feasible positions for phosphorylations. New methods could help to elucidate ways in which GLRs are tweaked in order to increase their sensitivity and precision. Employing updated techniques such as a liquid chromatography-mass spectrometry on a hybrid triple quadrupole linear ion trap mass spectrometer described by Williamson *et al.* (2006) could help to verify and quantify phosphorylation sites in GLRs. However, glycosylation events have to be determined, first, for example by affinity-based methods on SDS–PAGEs using saccharide-binding lectins. Then, structural analysis by exoglycosidase arrays followed by normal-phase high-performance liquid chromatography and mass spectrometry could provide information about further GLR-regulations. So far, little is known about possible binding partners of plant glutamate receptors and even if it will be very difficult to study an entire receptor complex, it is imperative to ascertain by which proteins they are affected and to what extent a regulation through other enzymes occurs. For this purpose, standard protein–protein interaction methods like affinity chromatography and coimmunoprecipitations could be combined with a recently published approach in which protein interactions were conducted

in biological liquids such as a cell lysate (Wienken *et al*. 2010). Aside from that, studies investigating the participation of GLRs in physiological pathways should utilise glutamate receptor overexpression lines more frequently in order to overcome at least partly the high GLR redundancy.

The more we will know about these basic components of signal transduction the better we will understand this fundamental signalling pathway not only in plants but in all organisms employing a related system.

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