Planta (2005) 222: 418–427
DOI 10.1007/s00425-005-1551-3

ORIGINAL ARTICLE

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# *AtGLR3.4*, a glutamate receptor channel-like gene is sensitive to touch and cold

Received: 8 December 2004 / Accepted: 16 March 2005 / Published online: 28 April 2005


**Abstract** The *Arabidopsis* genome encodes for 20 members of putative ligand-gated channels, termed glutamate receptors (GLR). Despite the fact that initial studies suggested a role for GLRs in various aspects of photomorphogenesis, calcium homeostasis or aluminium toxicity, their functional properties and physiological role in plants remain elusive. Here, we have focussed on *AtGLR3.4*, which is ubiquitously expressed in *Arabidopsis* including roots, vascular bundles, mesophyll cells and guard cells. *AtGLR3.4* encodes a glutamate-, touch-, and cold-sensitive member of this gene family. Abiotic stress stimuli such as touch, osmotic stress or cold stimulated *AtGLR3.4* expression in an abscisic acid-independent, but calcium-dependent manner. In plants expressing the $Ca^{2+}$ -reporter apoaequorin, glutamate as well as cold elicited cytosolic calcium elevations. Upon glutamate treatment of mesophyll cells, the plasma membrane depolarised by about 120 mV. Both glutamate responses were transient in nature, sensitive to glutamate receptor antagonists, and were subject to desensitisation. One hour after eliciting the first calcium signal, a 50% recovery from desensitisation was observed, reflecting the stimulus-induced fast activation of *AtGLR3.4* transcription. We thus conclude that AtGLR3.4 in particular and GLRs in general could play an important role in the $Ca^{2+}$ -based, fast transmission of environmental stress.

**Keywords** Calcium · Cold · Glutamate · Receptor · Touch

**Abbreviations** GLR: Glutamate receptor · DNQX: 6,7-dinitroquinoxaline-2,3-dione · CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione · MNQX: 5,7-Dinitro-1,4-dihydro-2,3-quinoxalinedione · GABA: $\gamma$ aminobutyrate · CHX: Cycloheximide

Data deposition: The sequences referred to in this paper have been deposited in the GenBank database (accession no. AF167355.1 [AtGLR3.4])

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## Introduction

Ionotropic glutamate receptors (iGluRs) represent a class of cation-selective, ligand-gated ion channels mediating excitatory synaptic transmission in nerve cells (Madden 2002). In animals, binding of the agonist glutamate leads to channel opening and, depending on the electrochemical gradients and channel selectivity, to membrane depolarisation and/or $Ca^{2+}$ entry. In plants, glutamate together with glutamine represent downstream metabolites during N-assimilation. Based on the finding that reduced N-sources such as ammonium, glutamate or glutamine negatively feed back on the transcription and the activity of nitrate transport systems, a role for these amino acids in the C/N-sensing mechanism is discussed (Coruzzi et al. 2001, and references therein). Using an antisense approach, it was shown by Kang and Turano (2003) that the *Arabidopsis* glutamate receptor AtGLR1.1 is indeed involved in C/N

sensing. Germination of anti-*AtGLR1.1* plants was inhibited by excess sucrose but could be restored in the presence of nitrate. In addition, molecular and biochemical analyses provided evidence for the regulation of C- and N-metabolic enzymes, depending on AtGLR1.1 activity. Based on the inhibition of light-induced hypocotyl shortening and the reduction of chlorophyll accumulation by the animal iGluR antagonist DNQX (6,7-dinitroquinoxaline-2,3-dione), initial studies suggested a role for plant glutamate receptors in light signal transduction pathways (Lam et al. 1998). In the studies that followed, the ionotropic glutamate receptor (iGluR) agonist BMAA [S(1)-$\beta$-methyl-$\alpha$, $\beta$-diaminopropionic acid] was shown to increase hypocotyl elongation and to inhibit cotyledon opening in the light (Brenner et al. 2000). A possible role in $Ca^{2+}$ homoeostasis for AtGLR2.1 was presented in plants overexpressing this particular GLR (Kim et al. 2001). In line with this finding, Dennison and Spalding (2000) could show that *Arabidopsis* root cells exhibit transient membrane depolarisations and changes in cytosolic calcium in response to glutamate. Recently, Sivaguru et al. (2003) proposed the involvement of plant GLRs in the context of aluminium toxicity. Root growth is severely impaired by soil contaminating aluminium and coincides with the depolymerisation of cortical microtubules of root cells. Both, inhibition of root growth and microtubule depolymerisation were mimicked by glutamate application but could be prevented by the calcium channel blocker gadolinium or iGluR antagonists. Anion channel blockers suppressed aluminium—but neither glutamate-induced membrane depolarisations nor microtubule depolymerisation. Thus, the authors suggested a role for an Al-sensitive anion channel as an efflux pathway for GLR ligands as a component of the plant responses to aluminium. Finally, using transgenic plants expressing apoaequorin to monitor changes in cytosolic calcium, Dubos et al. (2003) provided evidence for glycine to act synergistically together with glutamate in ligand-evoked calcium signals in plants.

Focussing on the ubiquitously expressed *Arabidopsis AtGLR3.4* gene, the present study aims on the elucidation of glutamate-based signalling networks in mesophyll cells of *Arabidopsis* leaves. Molecular studies based on the quantitative transcript analyses provided evidence for a role of $Ca^{2+}$ -dependent steps in the transcriptional regulation of *AtGLR3.4* by mechanical stress. Using Aequorin-based cytosolic calcium measurements as well as electrophysiological techniques, we followed the time-course of changes in cytosolic calcium and membrane voltage in this cell type in response to glutamate. Thereby, we found that mesophyll cells exhibited glutamate-induced, desensitising calcium and membrane-potential transients. Based on our observations that glutamate induced calcium signals were blocked by GluR-receptor antagonists and recovery from desensitisation seems to require de novo protein synthesis, a possible role of this ligand-gated receptor channel in touch- or cold-dependent signalling pathways is discussed.

## Materials and methods

### Plant material and growth conditions

*Arabidopsis thaliana* L. cv Col-0 suspension cultured cells were grown in growth medium (MS medium containing 2% sucrose, 28 mg·l$^{-1}$ $FeSO_4$ and 0.5 mg·l$^{-1}$ 2,4-D at pH 5.7) at 24°C on a shaker under white light with 11 µmol $m^{-2}$ ·$s^{-1}$. The cells were used for experiments after 3 days subcultivation. *Arabidopsis thaliana* L. cv Col-0 seedlings were grown on MS medium for 14 days under short-day conditions (light for 7 h with 22°C and dark for 17 h at 16°C). Immediately after harvesting, seedlings or cell cultures were frozen in liquid nitrogen and stored until use at −84°C. *Arabidopsis thaliana* plants for cytosolic calcium and membrane potential measurements were grown 4–6 weeks under short-day conditions on soil. The *Arabidopsis thaliana* wildtype seeds were provided by Lehle Seeds (Round Rock, Texas, USA)

### Isolation of guard cell (GC) and mesophyll cell (MC) protoplasts

Epidermal peels (for GC protoplasts) and epidermal-free fully developed rosette leaves (for MC cells) were incubated for 2 h (GC) or 20–30 min (MC) in enzyme solutions, containing 0.8% (w/v) cellulase (Onozuka R-10), 0.1% pectolyase (Sigma), 0.5% bovine serum albumin, 0.5% polyvenylpyrrolidone, 1 mM $CaCl_2$ and 10 mM Mes/Tris (pH 5.6). Osmolarity of the enzyme solution was adjusted to 540 (GC) or 400 mosmol $kg^{-1}$ (MC) with D-sorbitol. Protoplasts released from guard cells or mesophyll tissues were filtered through a 100 µm nylon mesh and washed twice in 1 mM $CaCl_2$ buffer (osmolarity 540 for GC or 400 mosmol $kg^{-1}$ for MC, pH 5.6).

### Quantitative reverse transcription (RT)-PCR

Total RNA from *Arabidopsis* seedlings and suspension-cultured cells was isolated with the RNeasy Plant Mini Kit (Qiagen) followed by a 2-fold mRNA selection with the Dynabeads mRNA Direct kit (Dynal, Oslo, Norway), to minimize DNA contaminations. First strand cDNA was prepared by using Superscript RT (GIBCO BRL) and diluted for RT-PCR 20-fold in water. PCR was performed in a LightCycler with the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals). The following GLR3.4-specific primers were used: GLR3.4fwd (5′-AGG GCA AGA GTT CAC A-3′) and GLR3.4rev (5′-CCG CAC TTT CTG GTA G-3′). To detect contaminations of genomic DNA, the primers were designed to flank an

intron. cDNA quantities were calculated by using Lightcycler 3.1 (Roche). All quantifications were normalized to actin cDNA fragments amplified by ACTfwd (5′-GGT GAT GGT GTG TCT-3′) and ACTrev (5′-ACT GAG CAC AAT GTT AC-3′). All kits were used according to the manufacturer's protocols.

### Transient expression of AtGLR3.4::GFP in onion epidermis peels

In order to localise AtGLR3.4, the cDNA with a mutated stop codon was cloned into pCR II TOPO (Invitrogen) downstream of a 35S promoter, mGFP4 was cloned c-terminal in frame of the cDNA to get a AtGLR3.4::GFP fusion protein. The construct was transiently expressed in onion epidermis peels using helium driven particle bombardment. 1.2 µm tungsten particles (Bio-Rad) were coated with DNA according to the manufacturer's directions.

### Glucuronidase (GUS) assay

The *AtGLR3.4* promoter was cloned into the pCRII-TOPO-TA vector (Invitrogen) as an 1508 bp PCR fragment. The subcloning was performed as an SstI/BamHI-compatible fragment into the pVKH-GUS-pA vector containing the uidA gene (GUS).

GUS histochemical staining was performed on whole 14-day-old seedlings grown on agar plates. Plants were harvested in a fixing solution (50 mM phosphate buffer, pH 7, 2% paraformaldehyde and 0.2% glutaraldehyde). Seedlings were rinsed with 50 mM phosphate buffer, pH 7. After vacuum infiltration with GUS staining solution (100 mM $NaH_2PO_4$, pH 7; 10 mM EDTA; 0.05% Triton X-100; 0.5 mg $ml^{-1}$ X-Gluc) for 5 min each, seedlings were incubated overnight at 37°C in the dark. The tissue was cleared by treatment with 70% ethanol and analysed by light microscopy.

### Luminescence measurements

Stimulus-induced cytosolic $Ca^{2+}$ -signals were measured in leaf segments of 4–6-weeks-old *Arabidopsis* plants expressing cytosolic apoaequorin (Knight et al. 1996; Baum et al. 1999). For reconstitution, a leaf segment with detached epidermis was floated overnight on 200 µl water or 2.5 mM Mes-Tris, pH 6.0, 0.1 mM $CaCl_2$ in the presence of 2.5 µM normal coelenterazine (Nanolight technologies, Pinetop, AZ, USA). The luminescence of the leaf section was determined using a cooled photomultiplier-based chemoluminometer (model 9829A, Thorn EMI, Electron Tubes Ltd., Ruislip, Middlesex, UK). Leaf sections were placed into a 3.5-ml cuvette (Sarstedt, Nümbrecht, Germany), and stimuli were given via a syringe through a luminometer port. To determine the relative luminescence $L/L_{max}$, after each experiment the aequorin was discharged by adding 1 M $CaCl_2$, 10% ethanol-solution. The relative luminescence was determined from the ratio of the actual luminescence and the total luminescence emitted from the probe and plotted as a function of time. For a 5 mM glutamate stimulus, 200 µl of 2.5 mM Mes-Tris, pH 6, 0.1 mM $CaCl_2$, 10 mM K-glutamate were added to the probe. In studies on recovery from desensitisation, the buffer after the first glutamate-stimulus was removed, and replaced by 200 µl of glutamate-free buffer.

### Membrane potential measurements

Mesophyll preparations were obtained from the rosette leaves of 8- to 10-week-old plants, the lower epidermis was removed to gain direct access to the spongy parenchyma cells. The peeled leaf-pieces were glued to microscope slides with Medical Adhesive B Liquid (Aromano Medizintechnik, Duesseldorf, Germany), afterwards the preparations were left in standard bath solution (5 mM KCl, 1 mM $CaCl_2$ and 5 mM Mes/Bis–Tris–propane, pH 6) for at least 3 h to recover. The microscope slides, with leaf tissue, were mounted in experimental chambers (volume $\sim$ 0.6 ml), which was continuously perfused at a rate of 3 ml $min^{-1}$ with standard bath solution. Glutamate was added to the standard bath solution at indicated concentrations, here $Cl^-$ was lowered to the same extend to keep the $K^+$ concentration constant. Electrodes were pulled from borosilicate-glass capillaries (Hilgenberg, Malsfeld, Germany, ø outside 1.0 mm, ø inside 0.58 mm), filled with 300 mM KCl and had a tip-resistance ranging from 30–50 MΩ. Cells were impaled using a micromanipulator (type 5171, Eppendorf, Hamburg, Germany) combined with a piezo translator (P-280.30, Physik Instumente, Waldbronn, Germany). The electrodes were connected via an Ag/AgCl halfcell to a micro electrode amplifier (VF-102, Bio-Logic, Claix, France) connected to a headstage with an input impedance of $10^{11}$ Ω. The free running membrane potential of mesophyll cells was recorded on a chart recorder and digitised afterwards.

## Results

### AtGLR3.4 is ubiquitously expressed and localises to the plasma membrane

Despite the striking phylogenetic and structural relationship with their animal orthologs (Lacombe et al. 2001; Chiu et al. 2002), little is known about the physiological role of plant glutamate receptors: in order to get a first overview about the distribution of these putative ligand-gated receptor channels in *Arabidopsis*, we followed GLR transcripts by RT-PCR. Thereby, we found all members of the GLR1 and GLR3 subfamily to be expressed in seedlings and suspension-cultured cells (data not shown). *AtGLR3.4* was expressed to a similar level in both systems and was therefore chosen for further anal-

yses. We employed ''real-time'' RT-PCR, to compare the level of transcripts in different organs, tissues, and cell types. By quantitative PCR following reverse transcription of mRNA (QRT-PCR), we detected *AtGLR3.4* mRNA in all tissues tested including protoplasts derived from mesophyll or guard cells (Fig. 1a). When analysing several independent transgenes expressing the *AtGLR3.4* promoter fused to the *uidA* gene, we found strong ß-glucuronidase (GUS) activity in cotyledons and roots of young seedlings (Fig. 1b, upper panel, left). In adult plants, GUS staining was visible in mesophyll cells of fully developed leaves and co-localised with vascular bundles and hydathodes (Fig. 1b, right). In addition, weak expression was visible in root hairs, root epidermis and cortex cells (Fig. 1b, lower panel, left). Thus, At *GLR3.4* shows a broad expression pattern with highest transcript levels in leaves and stem.

In order to follow the sub-cellular distribution of this receptor-like channel protein, the *AtGLR4.3* coding sequence was translationally fused to the Green Fluorescent Protein (GFP) and transiently expressed in onion epidermal cells (Fig. 1c). Thereby, we could localise GFP fluorescence to the plasma membrane (Fig. 1c, upper), while control cells, expressing a translational fusion of the vacuolar potassium channel TPK1 (KCO1) and GFP, exhibited fluorescence at the tonoplast (Fig. 1c, lower panel; Becker et al. 2004).

## Mechanical stress and cold induce AtGLR3.4 expression

In response to touch or wounding leaves of 4-weeks-old *Arabidopsis* plants expressing the GUS gene under control of the *AtGLR3.4* promoter exhibited distinct GUS

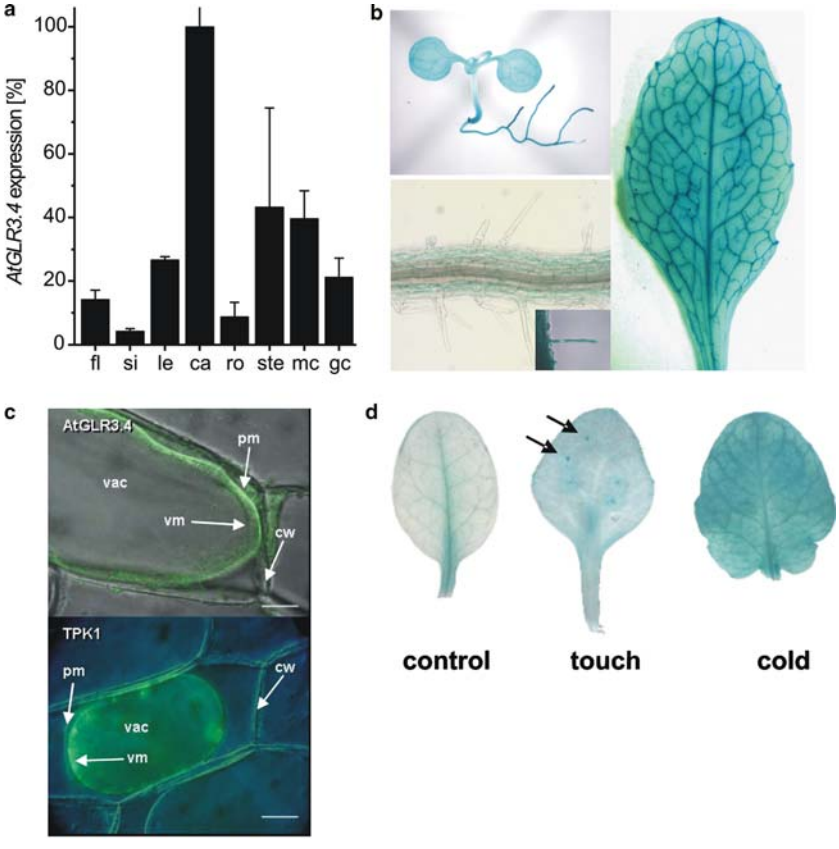


**Fig. 1 a–d** Ubiquitous expression and plasma membrane localisation of *AtGLR3.4* **a** Quantitative real-time RT-PCR analysis on cDNA derived from different organs or tissues. Expression of *AtGLR3.4* was detected in flowers (*fl*), siliques (*si*), leaves (*le*), cauline leaves (*ca*), roots (*ro*), stem (*ste*), as well as protoplasts of mesophyll (*mc*) and guard cells (*gc*). The relative comparison between different tissues revealed the ubiquitous expression of the *AtGLR3.4* gene (mean ± SD, $n = 3$). **b** GUS staining of transgenic T2 plants transformed with *AtGLR3.4Pr::GUS*. Blue staining in 2-weeks-old seedlings of *A. thaliana* shows AtGLR3.4 promoter activity in primary leaves and roots (*upper panel*, *left*). A close-up of root tissues indicates AtGLR3.4 in root hairs, root epidermis and cortex cells (*lower panel*, *left*). Mature plants exhibit GUS activity along the vascular tissue and mesophyll cells of fully developed leaves (*right panel*). **c** Subcellular localisation of an AtGLR3.4::GFP fusion protein following transient expression of the respective cDNA construct in onion epidermal cells (*upper panel*). Cells plasmolysed with 0.5 M $KNO_3$ exhibit GFP fluorescence at the plasma membrane and co-localise with filaments of Hecht, still attached to the cell wall. In contrast vacuolar potassium channel TPK1 (KCO1) localises to the tonoplast in onion epidermal cells transiently transformed with the corresponding TPK1-GFP cDNA fusion; plasma membrane (*pm*), cell wall (*cw*), vacuolar membrane (*vm*), and vacuole (*vac*). Bars represent 10 µm in the upper panel and 20 µm in the lower. **d** Touch and cold induce *AtGLR3.4* expression. Leaves from T2 plants expressing the *AtGLR3.4Pr::GUS* fusion revealed high GUS activity when leaves were touched with blunt-ended needles (*middle*) or subjected to a cold treatment at 4°C for 1 h (*right*)

activity in cells surrounding the perforation zones (Fig. 1d, middle). Upon a sudden drop in temperature from 20°C to 4°C for 1 h leaves showed uniform GUS activity (Fig. 1d, right), suggesting that physical forces seem to promote *AtGLR3.4* expression. In support of this hypothesis, we found *AtGLR3.4* transcripts to increase three- to six-fold when subjecting 2-weeks-old *A. thaliana* seedlings to touch or cold (Fig. 2a). Similarly, suspension cultured cells exhibited increased *AtGLR3.4* transcripts in response to osmotic stress imposed by addition of 660 mosmol sorbitol (Fig. 2a), a treatment known to increase endogenous ABA levels (Iuchi et al. 2001). To test whether the stress signals were sensed via abscisic acid (ABA), we treated *Arabidopsis* seedlings with 20 μM of this stress hormone. ABA, however, did not affect *AtGLR3.4* transcription, excluding this hormone as a transducer of physical stress into AtGLR3.4 gene activation (Fig. 2a). When glutamate was applied to *Arabidopsis* seedlings *AtGLR3.4* mRNA increased about 3–5 fold (Fig. 2a and b). Likewise γ-amino-butyric acid (GABA), malate and aspartate as well as membrane-permeable, weak acids (e.g. acetate) were able to induce *AtGLR3.4* expression. This indicates that in addition to cold and touch, cytoplasmic acidosis could serve as an additional signal, reflecting stress conditions and thus inducing *AtGLR3.4* expression.

To follow the time course of *AtGLR3.4* expression in response to cold in more detail, we shifted 2-weeks-old *Arabidopsis* seedlings from ambient temperatures (20°C) for 5 min to 4°C followed by an up to 2 h recovery period at again 20°C. Analyses of cold-treated seedlings revealed a doubling of *AtGLR3.4* message already after 5 min at 4°C (Fig. 2c, t = 0). During the subsequent recovery from cooling, *AtGLR3.4* transcripts reached a maximum at 15 min to drop again during the following 2 h, indicating that transcriptional activation of

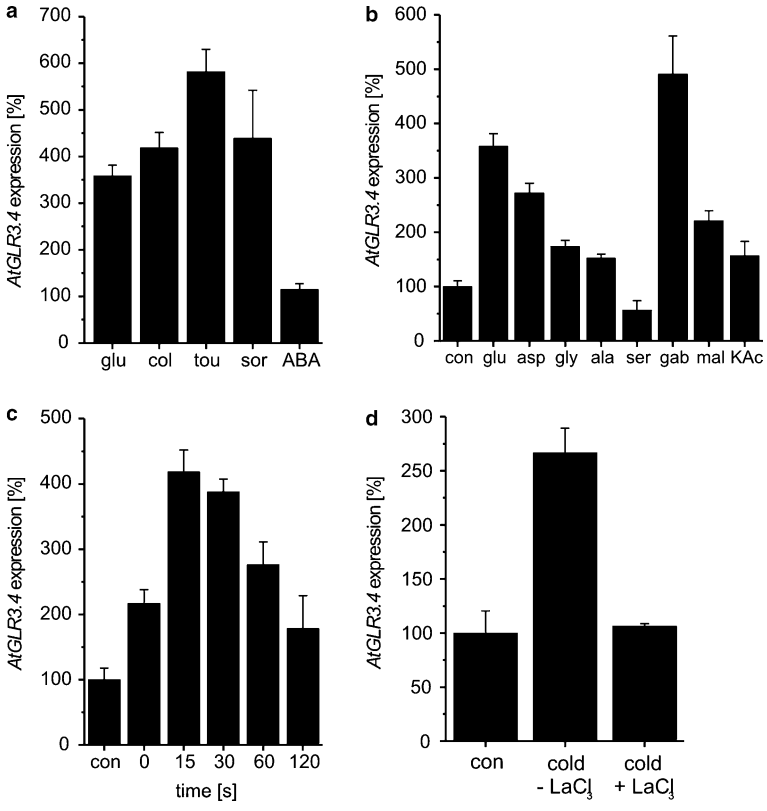


**Fig. 2 a–d** Stress stimuli induce *AtGLR3.4* transcription independent of ABA. **a** Quantitative RT-PCR analysis of *AtGLR3.4*-expression in response to various stress stimuli. 2-weeks-old, filter grown *Arabidopsis* seedlings were subjected to cold (*col*) or touch (*tou*) as described above or incubated for 1 h with either 20 mM glutamate (*glu*) or 20μM ABA (ABA). Sorbitol (sor) was applied to suspension-cultured cells at 660 mosmol $kg^{-1}$ for 1 h. **b** *AtGLR3.4* expression in suspension-cultured cells in response to external amino acids (*glu*, glutamate; *asp*, aspartate; *gly*, glycine; *ala*, alanine; *ser*, serine), γ-amino-butyric acid (*gab*), malate (*mal*) and potassium actetate (*KAc*) as revealed by quantitative RT-PCR analysis. Stimuli were applied at a concentration of 20 mM, and all solutions were adjusted to pH 5.7. **c** Cold induced *AtGLR3.4* expression is fast and transient. Kinetics of cold induced *AtGLR3.4* expression as revealed by quantitative RT-PCR. *Arabidopsis* seedlings were subjected to a 5 min cold stimulus at 4°C followed by a recovery period at ambient temperatures for the duration indicated. **d** Lanthanum blocks the cold induced *AtGLR3.4* expression. Suspension cultured cells were stressed by cold treatment for 5 min at 4°C in the absence or presence of 0.5 mM $LaCl_3$, respectively; mRNA was isolated after 15 min of recovery at ambient temperatures. If not stated otherwise RNA was isolated 20 min following the individual treatments, converted to cDNA, and subjected to quantitative real-time PCR; untreated seedlings and cells served as controls (con) and were set to 100%. Data represent mean values of at least three-independent experiments (mean ± SE, $n \geq 3$)

*AtGLR3.4* is fast and transient in nature. Cold activation of the receptor-channel gene, however, was prevented when the $Ca^{2+}$ -channel blocker lanthanum was present in the incubation medium of suspension-cultured cells (Fig. 2d). This suggests a calcium-dependent step in the transcriptional regulation of the *AtGLR3.4* gene.

## Glutamate induces membrane depolarisations and $Ca^{2+}$ transients in mesophyll cells

By monitoring the free-running membrane potential, we aimed to study the effect of putative receptor ligands on the plasma membrane potential of AtGLR3.4 expressing mesophyll cells. When mesophyll cells of rosette leaves from 3- to 4-weeks-old *Arabidopsis* plants were impaled with microelectrodes, an average resting potential of $-163 \pm 6$ mV ($\pm$SE, $n = 15$) was measured (Fig. 3). Upon stimulation with as low as 50 µM glutamate, mesophyll cells responded by a weak and transient depolarisation (Fig. 3, $\Delta E_m = 11 \pm 4$ mV $\pm$SE, $n = 6$). Rising the stimulus concentration to 500 µM glutamate induced a strong, transient depolarisation by $121 \pm 8$ mV ($\pm$SE, $n = 15$), which decayed to resting potential values ($-148 \pm 7$ mV $\pm$SE, $n = 15$) within 2 min (Fig. 3).

Following the first membrane potential transient, a further glutamate application within 8–24 min after glutamate removal, resulted in transients of largely reduced amplitude (Fig. 3, $\Delta E_m = 24 \pm 3$ mV $\pm$SE, $n = 15$). These results show that glutamate-induced membrane depolarisations were dose- or threshold-dependent and desensitised in the presence of the stimulus, a behaviour very similar to that of iGluR in animal membranes (Madden 2002).

To test the involvement of $Ca^{2+}$ signals in the mesophyll cell responses to glutamate, we took advantage of *Arabidopsis* plants constitutively expressing the

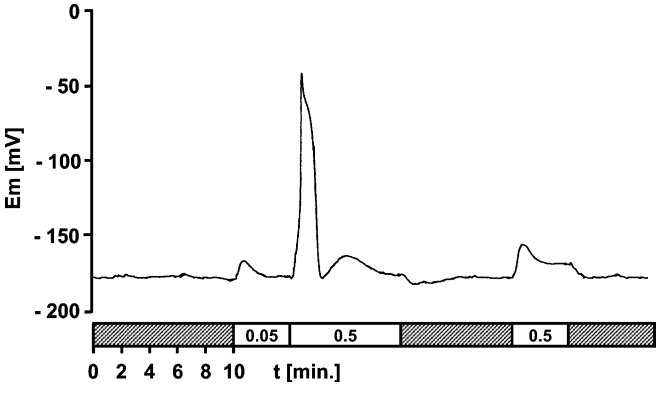


**Fig. 3** Glutamate elicits transient, desensitising membrane voltage depolarisations mesophyll cells. Glutamate-induced depolarisations of the mesophyll cell membrane. The free running plasma membrane voltage of a mesophyll cell was recorded with an intracellular microelectrode. Glutamate was given at concentrations of 0.05 and 0.5 mM as indicated by the bar below the trace. Similar results were obtained in 15-independent experiments. *Note*, that depolarisations increased in a concentration dependent manner, were transient in nature and desensitised

$Ca^{2+}$ reporter apoaequorin in the cytosol (Knight et al. 1995; Baum et al. 1999). An amount of 5 mM glutamate, when applied to epidermis-free leaves, induced a transient increase in cytosolic calcium concentration, as indicated by the increase in relative luminescence (Fig. 4a). Following stimulus onset, calcium transients reached a peak after 4 s, which decayed within 20 s (Fig. 4a and 5c). The observed calcium transients were dose-dependent (Fig. 4b) and, well in agreement with physiological apoplastic glutamate concentrations (Stitt et al. 2002), an apparent $K_M$ of 2 mM was calculated from the glutamate induced luminescence peak (Fig. 4b). Among different stimuli tested, highest calcium signals could be elicited by glutamate (Fig. 4c). Glycine and alanine were capable of generating calcium transients too, reaching 48% and 25% of the glutamate signal, respectively (Fig. 4c). In contrast, KCl, aspartate, glutamine, or γ-aminobutyrate (GABA) failed to generate calcium signals in mesophyll cells. Although of lower amplitude, protoplasts isolated from Aequorin expressing mesophyll cells fired calcium transients in response to glutamate as well (Fig. 4d). Thus, cell wall and turgor seem not to be essential for generating glutamate-mediated $Ca^{2+}$ transients, but may influence its amplitude. In order to test whether plasma membrane localised plant glutamate receptors could generate the glutamate evoked changes in cytosolic calcium, we recorded calcium changes in the presence of the iGluR antagonists DNQX (6,7-dinitroquinoxaline-2,3-dione), CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione), MNQX (5,7-Dinitro-1,4-dihydro-2,3-quinoxalinedione) or the non-specific calcium channel blocker $La^{3+}$. Extracellularly applied $La^{3+}$ inhibited the glutamate evoked calcium signals (Fig. 5a), suggesting that influx of apoplastic calcium ions contributed to the observed calcium signals. In line with glutamate receptor mediated $Ca^{2+}$ currents, the antagonists DNQX, CNQX, and MNQX efficiently reduced the glutamate dependent calcium signals (Fig. 5b) in mesophyll cells.

A second glutamate treatment, right after the first stimulus, did not cause an additional $Ca^{2+}$ response (data not shown). Reminiscent of the electrophysiological studies, mesophyll cells recovered within 1 h to about 40–50% of the initial glutamate sensitivity when glutamate was removed after the first $Ca^{2+}$ response (Fig. 5c). Thus, in AtGLR3.4 expressing mesophyll cells, glutamate-induced changes in membrane potential and cytosolic $Ca^{2+}$ concentration share a dose-dependent response to the stimulus, and a desensitisation. To study whether the recovery from desensitisation would depend on de novo synthesis of GLR proteins induced by glutamate (Fig. 2), we pre-incubated the epidermis-free leaves in the presence of 70 µM CHX (cycloheximide), a well known translation inhibitor. A 60 min pre-treatment with CHX did not affect the glutamate-evoked first $Ca^{2+}$-transient (Fig. 5c). In contrast, a second $Ca^{2+}$-response, following a 1 h glutamate-free interval, was completely abolished in the presence of CHX (Fig. 5c). These data indicate that the sensitivity of mesophyll cells

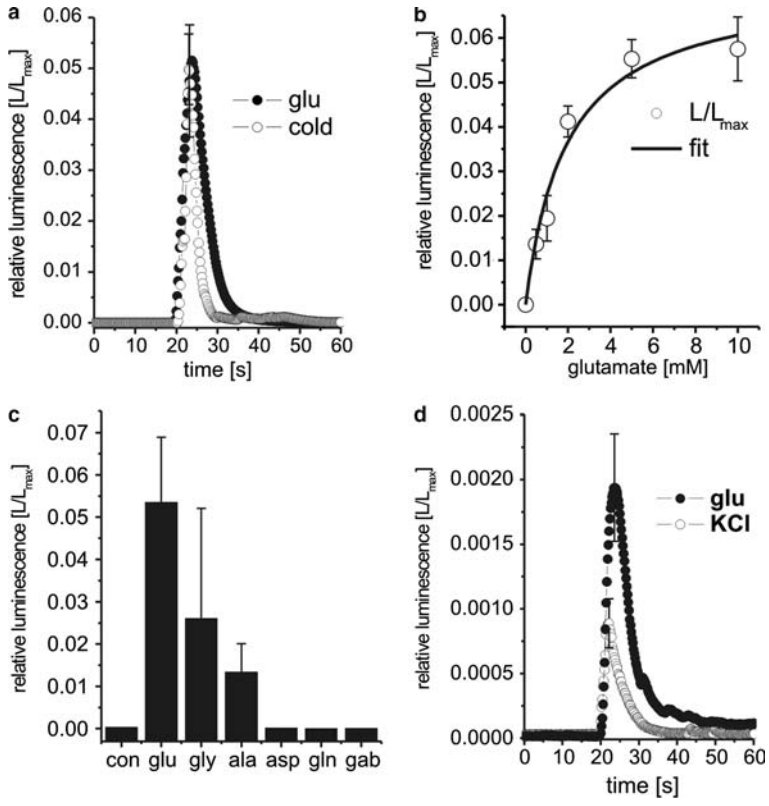


**Fig. 4 a–d** Glutamate induces $Ca^{2+}$ -transients in *Arabidopsis* leaves. **a** Transient changes in cytosolic $Ca^{2+}$ concentrations in leaf sections upon glutamate-stimulation. As a measure for changes in cytosolic $Ca^{2+}$ concentration, the relative luminescence of one leaf section with reconstituted aequorin was followed before and during application of 5 mM glutamate. Representative measurement from a data set with $n > 20$. Note that cold induced $Ca^{2+}$ changes of similar amplitude but of different kinetics. **b** Saturation of glutamate-induced $Ca^{2+}$ changes. Peak luminescence values as seen in (**a**) were determined and mean values plotted as a function of the glutamate concentrations applied ($n = 8$–24). Data were fitted by the Michaelis–Menten function revealing an apparent $K_m$-value of 2 mM glutamate. **c** Amino acid specificity of $Ca^{2+}$ -signals (10 mM each, KCl-control buffer (*con*), glutamate (*glu*), glycine (*gly*), alanine (*ala*), aspartate (*asp*), glutamine (*gln*), γ-amino-butyric acid (*gab*)). Amino acids were applied as K-salts and data were obtained as in (**a**). Maximum responses were averaged (mean ± SE, $n \geq 10$). **d** Transient changes in cytosolic $Ca^{2+}$ concentrations in mesophyll protoplasts upon glutamate-stimulation. Representative measurement (mean ± SE) of data as obtained in (**a**) from a data set with $n = 5$

to glutamate relies on a protein that has to be replaced by de novo synthesis after activation. It is tempting to speculate that this protein is the glutamate receptor itself, which in case of GLR3.4 resides in the plasma membrane.

## Discussion

The plant glutamate receptor family comprises a class of receptor-like channels of still unknown function (Lacombe et al. 2001; Davenport 2002; White et al. 2002; Chiu et al. 2002; Bouche et al. 2003). Following heterologous expression in *Xenopus* oocytes, AtGLR3.4 gave rise to voltage dependent inward currents, carried by various cations including $K^+$, $Na^+$, $Cs^+$, or $Ca^{2+}$ (data not shown). These currents, however, were not affected by application of glutamate to the bath perfusion, suggesting improper receptor protein folding, glycosylation or missing subunits in the functional receptor channel. *Arabidopsis thaliana* root cells, however, have been shown to respond to glutamate application by changes in cytoplasmic calcium and membrane voltage depolarisations (Dennison et al. 2000) indicating a role for glutamate as a signalling molecule in plants.

Here, we have shown that GLR genes are ubiquitously expressed in *Arabidopsis* organs among them, the *AtGLR3.4* gene encodes a plasma membrane localised protein which expression is highest in leaves and stem. The plasma membrane of AtGLR3.4 expressing mesophyll cells transiently depolarises in response to externally applied glutamate. This electrical response lasted for about 2 min and can be decomposed into a fast initial rise phase, a plateau and a repolarisation phase. The multiphase response is reminiscent of other transient phenomena, which involve ligand, $Ca^{2+}$, and voltage-gated channels. In *Chara*, the action potential is triggered by an initial $Ca^{2+}$ influx, which in turn activates calcium-dependent anion channels and depolarisation-activated potassium channels (Tazawa et al. 2003, and references therein). The time course of the glutamate-evoked changes in membrane voltage shown

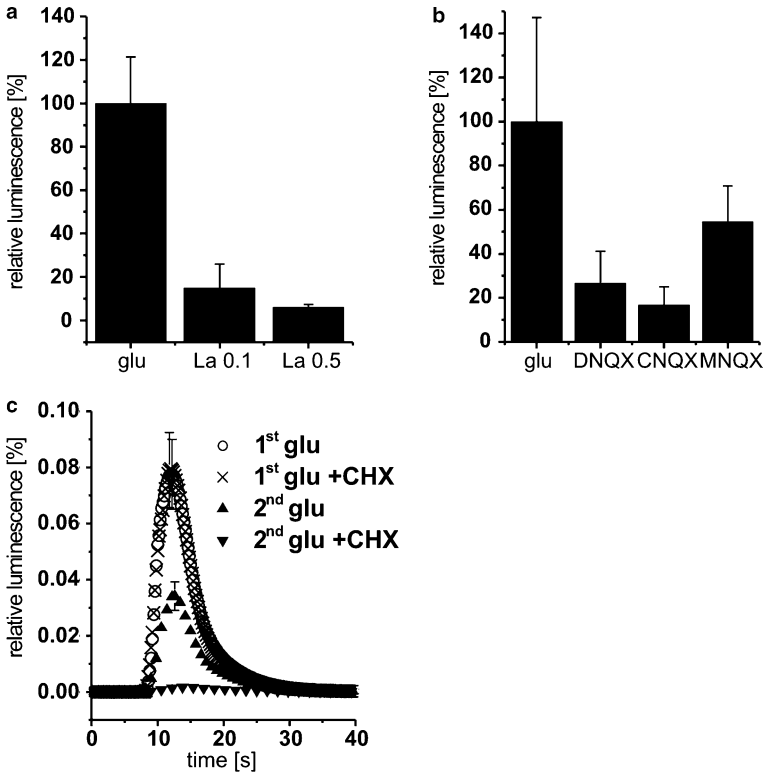


**Fig. 5** **a**–**c** Pharmacology and desensitisation of glutamate induced $Ca^{2+}$ signals. **a** Pharmacology of glutamate-dependent $Ca^{2+}$ signals. Calcium signals were induced by application of 5 mM glutamate in the absence of inhibitor (*glu*) and presence of 0.1 (La 0.1) or 0.5 mM (La 0.5) $LaCl_3$, respectively. **b** Antagonists of animal iGluR proteins block glutamate induced $Ca^{2+}$ transients. $Ca^{2+}$ signals were induced by application of 1 mM glutamate in the absence (*glu*) and presence of either 0.5 mM DNQX, CNQX, or MNQX. Data in (**a**) and (**b**) were determined as described above (**a**), peak values averaged and expressed relative to the glutamate control (mean ± SE, $n \geq 5$). **c** Desensitisation of glutamate dependent $Ca^{2+}$ signals. Leaf segments were preincubated for 1 h with 70 µM CHX and the first $Ca^{2+}$ signals (first glu) were triggered by application of 10 mM glutamate. A second $Ca^{2+}$ signal was again triggered with 10 mM glutamate following a recovery period of 1 h in the absence of glutamate and absence (second glu) or presence of CHX (second glu + CHX), respectively. Data represent average values of 7–15 independent experiments (mean ± SE, $n = 7$–15). Note, that a 1 h preincubation with CHX did not affect the first glutamate response

here suggests that cytoplasmic calcium changes preceded the observed membrane-potential transients, while a possible activation of $Ca^{2+}$ -dependent anion channels (Elzenga and Van Volkenburgh 1997) and depolarisation-activated $K^+$ channels (White et al. 1999) would represent downstream events. Using plants expressing the cytosolic $Ca^{2+}$ -reporter apoaequorin, we could indeed demonstrate that glutamate elicits cytoplasmic $Ca^{2+}$ transients, which peaked already within 4 s following the stimulus onset (Fig. 4a, b and 5c), supporting the idea that $Ca^{2+}$ influx may trigger the depolarisation phase, but that other channel types underlie the plateau- and repolarisation phase (Fig. 3).

Integrating the results obtained from RT-PCR, impalement studies and luminescence measurements into a current model, we suggest that apoplastic glutamate and/or glycine (Dubos et al. 2003) can act as potential ligands of plasma membrane localised receptors (Fig. 6). Glutamate binding to inactive receptors ($GLR_i$) would induce conformational changes rendering the receptor protein active ($GLR_a$), and $Ca^{2+}$ permeable. The fact that glutamate evoked cytoplasmic calcium transients were susceptible to iGluR antagonists (DNQX, CNQX and MNQX) and the non-selective cation channel blocker lanthanum ($La^{3+}$) provides evidence for plant glutamate receptors as calcium permeable pathways in *Arabidopsis* mesophyll cells (c.f. Dennison et al. 2000; Dubos et al. 2003). Although on a different time scale, both, the glutamate induced $Ca^{2+}$ changes and membrane potential depolarisations were of transient nature. This behaviour might be explained by a further conformational step in the receptor channel, when activated $GLR_a$ converted into a desensitised state ($GLR_d$) in the presence of the agonist. The desensitised state appears to be long lasting rendering *Arabidopsis* mesophyll cells insensitive to repetitive glutamate stimulation. Recovery from desensitisation seems to require de novo protein synthesis, since CHX-treated cells still responded to the first glutamate stimulation, but failed to regenerate glutamate susceptibility even after 1 h. The fact that cold was capable of inducing a calcium signal before the recovery of the glutamate response (data not shown) indicates that cold seems to address other calcium entry pathways. This finding is in agreement with the observed insensitivity of cold-induced $Ca^{2+}$

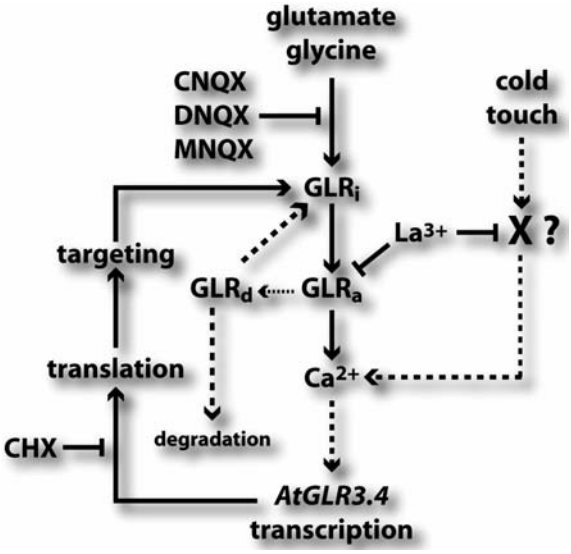


**Fig. 6** Schematic view of glutamate and cold induced *AtGLR3.4* expression. The current model suggests that plant GLRs represent $Ca^{2+}$ entry pathways at the plasma membrane of mesophyll cells. According to this working model, binding of glutamate and/or glycine would result in the activation of inactive GLRs ($GLR_I \rightarrow GLR_a$) and thus trigger GLR-mediated $Ca^{2+}$ influx followed by elevation of cytosolic calcium ions within a few seconds. Subsequently, activated GLRs ($GLR_a$) convert into a desensitised state ($GLR_d$) via a yet unknown mechanism. Thereby, further $Ca^{2+}$ influx is prevented and mesophyll cells enter a refractory phase characterised by glutamate insensitivity. Recovery from desensitisation seems to involve $Ca^{2+}$ -dependent transcriptional activation of the *AtGLR3.4* gene and subsequent de novo synthesis of new receptor channels brought by CHX-sensitive translational activity. Stress signals such as cold or touch seem to address different receptors and/or channels, but meet the glutamate pathway at the level of cytoplasmic $Ca^{2+}$ -rises and transcriptional activation of the *AtGLR3.4* gene

transients towards the competitive glutamate receptor antagonist DNQX (Dubos et al. 2003). Cold, touch, and glutamate activate the transcription of the *AtGLR3.4* gene in a $Ca^{2+}$ dependent but ABA-independent manner. Transcriptional activation of *AtGLR3.4* in response to e.g. cold was fast (5–15 min) and the CHX experiments suggest that translation of the putative receptor channel rather than transcription seems to represent the bottleneck for regeneration of glutamate sensitivity. Up to now the identity of cold or touch receptors are still scant, but an immediate rise in cytoplasmic $Ca^{2+}$ and activation of calmodulin-related touch genes (TCH) in the 1–5 min range represents a common response in both signal transduction pathways (Braam et al. 1997). Thus, cold and touch may meet the glutamate pathway on the level of the cytoplasmic $Ca^{2+}$ signal. Our finding, that a rise in cytosolic calcium is a prerequisite for *AtGLR3.4* gene activation is in agreement with this hypothesis. Thus touch and/or cold as positive regulators of *AtGLR3.4* gene activity could act as modulators to fine-tune the responsiveness of mesophyll cells to changes in glutamate and/or glycine. Although neither glycine nor glutamate have been recognised as typical signal metabolites in plants, there is increasing evidence that these metabolites exert their action on plant GLRs in a physiological context of changing environmental conditions. If plant, glutamate receptors would function as integrators of these stimuli the desensitisation process described here would stabilise the refractory phase preventing overstimulation of the system. To regain sensitivity the stimulus- and $Ca^{2+}$ -dependent activation of e.g. *AtGLR3.4* expression could provide a feed-forward loop for the synthesis of new receptor channels and subsequent incorporation into the plasma membrane.

**Acknowledgements** We thank K. Neuwinger and D. Giehl (University of Würzburg, Ger) for assistance during the $Ca^{2+}$ measurements. We are grateful to A. Trewavas (University of Edinburgh, UK) for supplying us with seeds of apoaequorin-expressing *Arabidopsis* plants. This work was funded by DFG grants and Körber European Science Award to RH, and by a DFG grant (SPP1108) to PD and DB.

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