Chapter 4

Pharmacological Studies with Specific Agonist and Antagonist of Animal iGluR on Root Growth in *Arabidopsis thaliana*

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Additional information is available at the end of the chapter

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Abstract

Ionotropic glutamate receptors (iGluRs) are a group of proteins with a high degree of sequence homology. At least 20 type of putative ionotropic glutamate receptor (iGluR)-like channels have been identified in *Arabidopsis thaliana*. To uncover the role of iGluR-like channels in plant root growth, we used a comprehensive set of compounds known to alter iGluR channels in the neurons. We found that Arabidopsis root system is highly sensitive to these compounds. iGluR competitive antagonists 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 6,7-dinitroquinoxaline-2,3-dione acted (DNQX) acts as a negative regulator of primary root and lateral root density. Continuous growth on antagonist also leads to impairment of root meristem size, which suggests that iGluR-like channels may play a role in meristem maintenance. However, application of iGluR agonists L-glutamate recovered Arabidopsis root growth. Taken together, these results suggest a correlation between the putative iGluR-like channel function and the alteration of root growth and development in the Arabidopsis roots.

Keywords: glutamate receptor, lateral root, Glu, calcium, DNQX, CNQX

1. Introduction

A mixture of organic and inorganic materials that makes uppermost layer of the earth in which plants grow is known as Soil. The parent mineral rock derives inorganic materials and is found in the form of sand, silt and clay. However, organic materials come from dead and decayed parts of bacteria, fungi, algae, protozoa and soil animals such as nematodes, earthworms, beetles and termites. The inorganic nitrogen dissolved in soil is vital for nutritional requirements of plants, and it can be directly used in the synthesis of amino acids,

peptides and proteins [1]. Plants absorb organic nitrogen from soil in the form of free amino acids [2, 3], which is derived mainly from decomposed organic matter and exudates produced by bacteria, fungus and living plants roots [4–9].

Among the 20 common amino acids, the six amino acids (glutamic acid, glutamine, aspartic acid, asparagine, alanine and histidine) are mainly dominated in the soil, and they cover approximately 80% of the total soil amino acid pool [10–12].

An agonist is an inducing ligand that can bind to and induce channel-linked receptors. On the contrary, antagonist is a type of receptor ligand that can block the agonist-mediated responses. Since ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels, binding of L-glutamate (Glu) will open gates and increase ions conductance. However, both agonists and antagonists of iGluRs share structural similarity with glutamate and bind to iGluRs at the same site where Glu binds [13]. Interestingly, it has been observed that major amino acids (glutamate, glycine, alanine, serine, asparagine, and cysteine) present in the rhizosphere are strong agonist for iGlurRs [14].

Previous studies indicate that plant GLRs are functional, and involved in various functions, such as photosynthesis [15, 16], abiotic stress [17, 18], as C/N balance [19], plant-pathogen interaction [20, 21], root morphogenesis [22–24], pollen tube growth [25] and regulate cellular calcium homeostasis [14, 20, 26–29]. Among studies with various cell types in plants, it was found that Glu induces intracellular Ca^{2+} current. Glu-induced rise in the intracellular Ca^{2+} level can be inhibited by the use of iGluRs antagonists, which are quinoxalinediones, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) [18, 27, 30]. Therefore, it was proposed that glutamate receptors (GLRs) can contribute to the network of Ca^{2+} signaling pathways in plant cells [16]. *Atglr1.2* knock-out mutant plants displayed abnormalities in pollen growth [25]. Further, Analysis of *Arabidopsis* GLR mutant, *atglr3.6*, reveals a major role of the plant GLRs in the regulation of plant root development [24]. As a signaling molecule, glutamate is regarded to be the major neurotransmitter in the mammalian central nervous system. The application of exogenous Glu can also alter root phenotype [31, 32], indicating a role for GLR signaling in plants. Additionally, MEKK pathways can alter the glutamate sensitivity at the root tip suggesting for a glutamate signaling pathway in plants [33, 34].

These days pharmacology-based functional study of ionotropic glutamate receptors in plants has become very popular and useful approach [17, 18, 27, 32, 35–37]. We used comprehensive set of compounds that have been found to contain a strong ability to modulate the activity of mammalian iGluRs. In the present study, we introduced Glu to study the possible role of plant GLRs in root development. To minimize the chance of multiple effects of Glu, we also used artificial agonists (NMDA and AMPA) and competitive antagonists (DNQX and CNQX) to the glutamate binding site on receptors. In animals, these artificial agonists and antagonists are reported only for specific effects via their impact on iGluR activities [38]. In our pharmacological-based study, we investigate how glutamate and iGluRs antagonists directly affect plant root growth and development.

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2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (Col-0) seedlings were used in different analyses on root development. All seed germination treatments were carried out at same half-strength Murashige and Skoog (MS) medium [39] at constant pH 5.8. The root elongation under various treatments was quantified using ImageJ program (http://rsb.info.nih.gov/ij/).

2.2. Chemicals

L-aspartic acid (Sigma, USA), L-glutamic acid, monosodium salt (Sigma, USA), N-Methyl-D-aspartate (NMDA; Sigma, USA), and 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid (AMPA; Sigma, USA) were dissolved in water, adjusted to pH 5.8 and filter sterilized. Both receptor antagonists, 6,7-dinitroquinoxaline-2,3-dione (DNQX; Sigma, USA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris, USA) were dissolved in organic solvent Dimethyl sulfoxide (DMSO) (Sigma, USA). All treatments were used with variable concentrations as indicated in the figure legends. In order to study the role of Glu in auxin balance inside the root cells, we used a synthetic auxin Naphthaleneacetic acid (NAA, Sigma) and a polar auxin transport inhibitor NPA (1-N-Naphthylphthalamic acid, Sigma) for treatments.

2.3. Seed sterilization

Prior to germination at growth media, seeds were first surface-sterilized in sodium hypochlorite in active chlorine. Sterilization was carried out in a hood cabinet, and aliquots of seeds were placed in Eppendorf tubes and treated with active chlorine for 1–2 h.

2.4. Plant growth condition

Arabidopsis seedlings for analysis were grown in sterile petri dishes using half strength MS medium and the plate was sealed using Micropore TM tape. After this, seeds were stratified in the dark at 4°C for 2–3 days to synchronize germination. Plates were then transferred to a growth chamber at illumination of 120–150 μmol/m^2 s continuous light and at temperature 22–23°C.

2.5. Laser scanning and light microscopy

Confocal microscopy was performed using a Zeiss LSM510 META Confocal Imaging System (USA). To observe the apical root meristem through confocal microscopy, roots were counterstained in propidium iodide (PI, Sigma) (10 μM) for 2–3 min, rinsed, mounted in dH_2O. Images were obtained by excitation with the Kr/Ar 488-nm laser line and emission was detected with a band-pass 500–550 nm filter.

2.6. Statistical analysis

Each experiment was repeated at least three times. Values are expressed as mean ± SD. The statistical significance was analyzed using Student's *t*-test analysis.

3. Results

3.1. iGluR agonists and antagonists alter root growth in Arabidopsis

We used a comprehensive set of compounds that have been found to modulate iGluRs. All treatments were performed with half strength of MS media [39] at a constant pH 5.8. The presence these compounds was observed to have a marked effect on root architecture of Arabidopsis. Both Glu and NMDA treatments had a stimulatory effect on primary root length (PRL) as well as lateral root density (LRD) in wild-type plants as compared to the non-treated plants. However, up to 10 days, AMPA showed a minor effect on root growth, but afterward, AMPA addition also nearly restored root growth of wild-type plants, making it visually indistinguishable from that of NMDA-treated plants (**Figure 1A** and **B**). These results indicated that glutamate receptor agonists likely interact with signaling pathways to control root growth in plants. Further, to test whether root growth was specific to natural iGluR ligands (Glu), we used another kind of neurotransmitter amino acid L-aspartate (Asp) [40]. Interestingly, after 12 days of growth, Asp treatment showed modest activity at inhibiting root growth and failed to increase lateral root formation when supplied at the same concentrations as Glu (**Figure 1A** and **B**). These results indicate that Glu and Asp have different activity in Arabidopsis root growth modulation and that the effects of Glu on root development are likely due to a specific effect of Glu rather than as a consequence of acidic behavior of amino acids.

To determine more closely the effects of plant iGluR-like receptor on the architecture of the Arabidopsis root system, wild-type Arabidopsis seedlings were germinated and grown on vertically oriented agar plates containing half strength MS medium supplemented with iGluR antagonists (DNQX and CNQX) alone or in combination of antagonists with Glu. As expected, our results show that both DNQX and CNQX drastically reduced root growth, and induced approximately similar kind of effects on root growth (**Figure 1C** and **D**). It was seen that the PRL approximately reduced by 64.65% and 69.24% and LRD by 76.1% and 76.55% (respectively for DNQX and CNQX treatment) (**Figure 1C** and **D**). To observe the effect of agonist and antagonist treatment together, we used naturally occurring agonist, Glu, to compete with DNQX and CNQX inhibitory actions [41]. It was observed that the external supplement of Glu (at 0.5 mM) successfully recovered the reduced root growth (both PRL and LRD) (**Figure 1C** and **D**). In summary, root growth was promoted by iGluR agonists, and use of iGluR antagonists (CNQX and DNQX) drastically reduced root growth and then, again subsequently recovered by addition of Glu suggesting molecular correlation. Since, these comprehensive set of compounds are called the great modulator of iGluRs in mammalian cells, our results suggested the involvement of Arabidopsis iGluR-like channel in root development.

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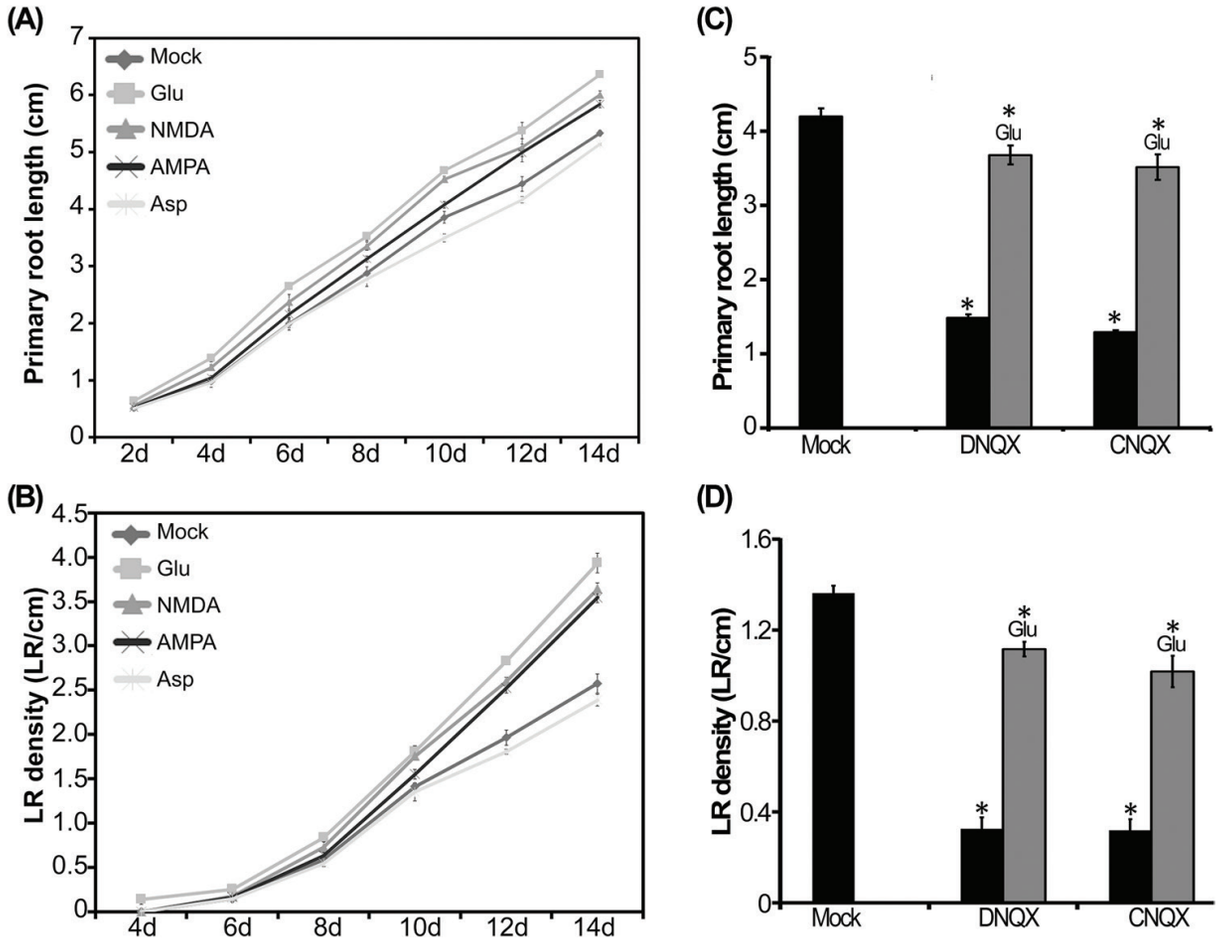

Figure 1. Variable effects of animal iGluR effector compounds on *Arabidopsis thaliana* root development. Arabidopsis (wild-type, Col-0 ecotype) seedlings were germinated on half strength of MS medium (MS/2, [39]) supplemented with 1% of sucrose and agar. Immediately after germination, different treatments were done in MS/2 basal media adjusted to pH 5.8 with NaOH. Time course for agonist treatments response in days 2–14 of longitudinal primary root growth (A) and LR density (B) represented as LRs per centimeter primary root of Col-0 after incubation with 0.5 mM of each glutamate (Glu), NMDA, AMPA and aspartate (Asp) individually. Antagonist's treatments were done in MS/2 basal media but control seedlings (Mock) were grown with equal volume of solvent (DMSO) as in DNQX (1 mM) and CNQX (1 mM) treated seedlings. Comparison of root growth under antagonist given alone (1 mM) or together in the treatment of 0.5 mM Glu. Root length (C) and LR density (D) of 11-day-old Col-0 seedlings. Values represent the mean of 15–18 measurements in triplicate and error bars represent ±SD. The statistical analysis were performed by Student's *t*-test ($P < 0.005$) indicated by asterisks.

3.2. Short-root growth in antagonist treated wild-type roots is contributed by reduced root meristem size

Previously we concluded that the glutamate receptor signaling may be involved in regulatory mechanisms in the control of root growth, indicating an essential role for plant GLRs in root meristem maintenance Therefore we analyzed cell division and meristem size among wild-type and antagonist-treated wild-type roots at different growth duration (4 and 6 days). However, since treatments of both antagonists induced similar kind of inhibitory effect on root growth, and thus we selected only one antagonist (DNQX) for further studies.

Interestingly, we observed that antagonist-treated wild-type root illustrated a smaller meristem size compared to wild-type (**Figure 2A** and **B**). Simultaneously, the number of meristematic epidermal cells (in a single file) was also significantly reduced in both 4 and 6-day-old roots of DNQX-treated wild-type plants (**Figure 2C**). Reduced meristem-enriched tissues in DNQX treated roots showed a putative vital contribution of putative *AtGLR* signaling in Arabidopsis root development.

QC surrounded with stem cells are pivotal in cell proliferation and meristem maintenance in root [42]. Thus we investigated the possibility of deformity in the stem cell niche which may result in impaired root growth after antagonist treatment. In confocal sections of propidium iodide (PI) stained roots (**Figure 3A** and **B**), we observed that in comparison to wild-type (four-celled QC), DNQX-treated wild-type roots were characterized by small dislocated columella cells with complicated-cellular-patterns. Altered columella root cap cells can also be

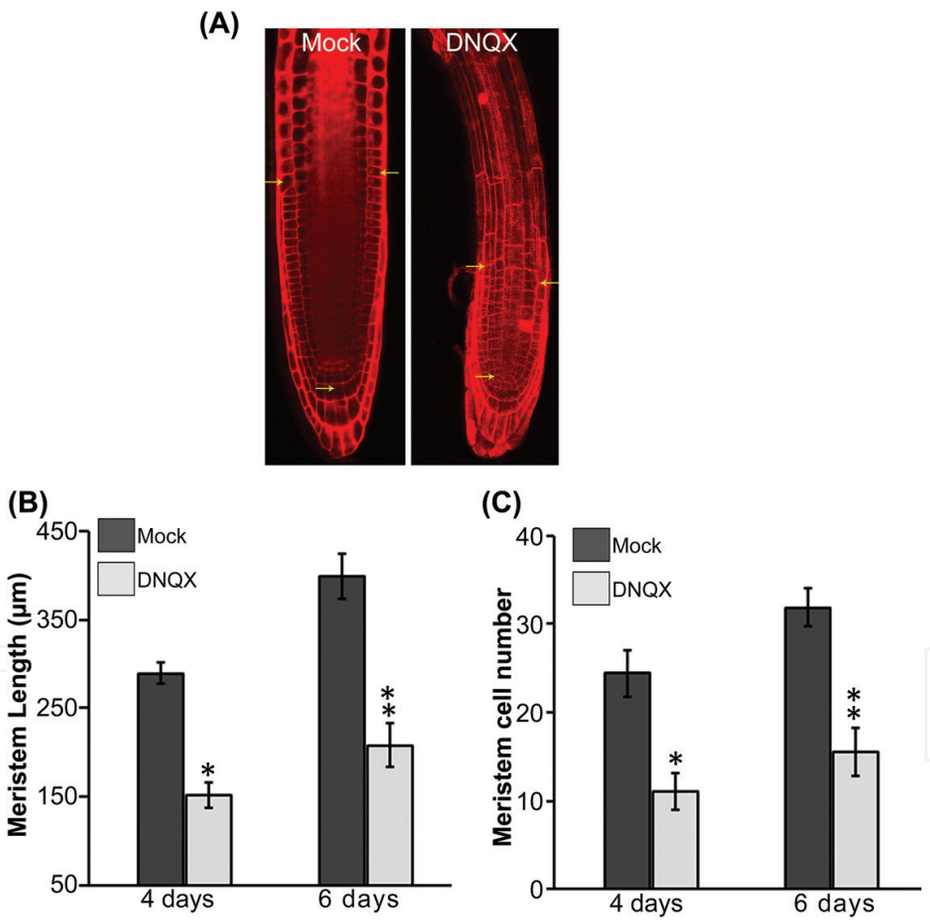

Figure 2. Putative AtGLR regulates meristematic activity in primary-root apical meristem. (A) Confocal microscopic images of PI-stained 4-day-old wild-type and DNQX treated wild-type root. The border of root apical meristem is indicated by arrows in PI-stained roots. The longitudinal distance between the quiescent center (QC, marked in lower arrow) and the first elongating cell is correspond to the root meristem length (B) and the number of meristematic epidermal cells in single file of cells in wild-type and DNQX treated wild-type root at various time points (4 and 6-day-old seedling) (C). Error bars represent SE ($n > 15$). Statistical significance in compared with wild-type were analyzed by Student's *t*-test ($P < 0.005$).

observed by Lugol staining of starch granule [43]. We found that DNQX treatment in wild-type approximately abolished the starch grains from amyloplast (**Figure 3C** and **D**).

3.3. Externally supplied Glu can rescue the EGTA-inhibited root phenotypes

There are many studies which showed that Arabidopsis AtGLRs engage in calcium homeostasis [27, 28, 30]. We investigated whether the induced root growth in Glu-treated seedlings was dependent on Ca^{2+}. Various concentrations of EGTA (a Ca^{2+} chelator) was added to MS/2 supplemented with 0.5 mM Glu. At both concentrations of EGTA (0.5 and 1.0 mM), root elongation was drastically inhibited in wild-type seedlings. However, supplement of external Glu partially recovered root growth inhibited by low amount of EGTA (**Figure 4**). Collectively, these data suggest a role for Ca^{2+} in AtGLRs signaling to control root growth.

Auxin has been recognized as a key regulator in root development [44, 45]. NPA is a drug that known for inhibition of polar auxin transport. An induction of cytosolic Ca^{2+} was observed after auxin application, indicating a strong correlations between Ca^{2+} and auxin signaling. Therefore, we investigated whether the higher root growth observed in the Glu-treated seedlings is linked to the auxin and calcium. To elucidate this, we investigated whether Glu and $CaCl_2$ are able to minimize the negative effect of NPA on Arabidopsis root growth. Interestingly, applications of Glu and $CaCl_2$ to NPA-treated wild-type seedling had restored the number of LR (**Figure 5A** and **B**).

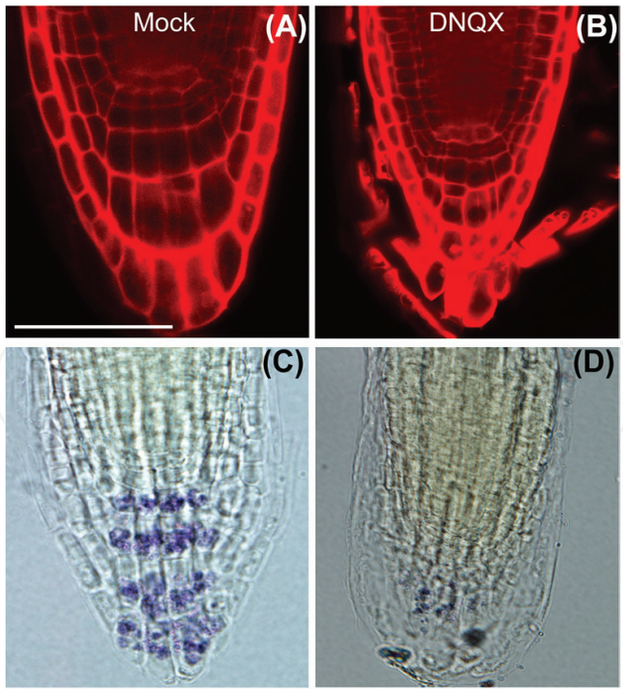

Figure 3. Putative AtGLR regulates meristematic activity in primary-root apical meristem. Statistical significance in compared with wild-type were analyzed by Student's *t*-test ($P < 0.005$), indicated by asterisk. (A–B) Confocal images of 4-day-old PI-stained wild-type and DNQX treated wild-type roots. Columella cells have abnormal cell divisions in DNQX treated wild-type roots. Wild-type and DNQX treated wild-type roots in Lugol staining (C–D). Scale bar: 100 μm.

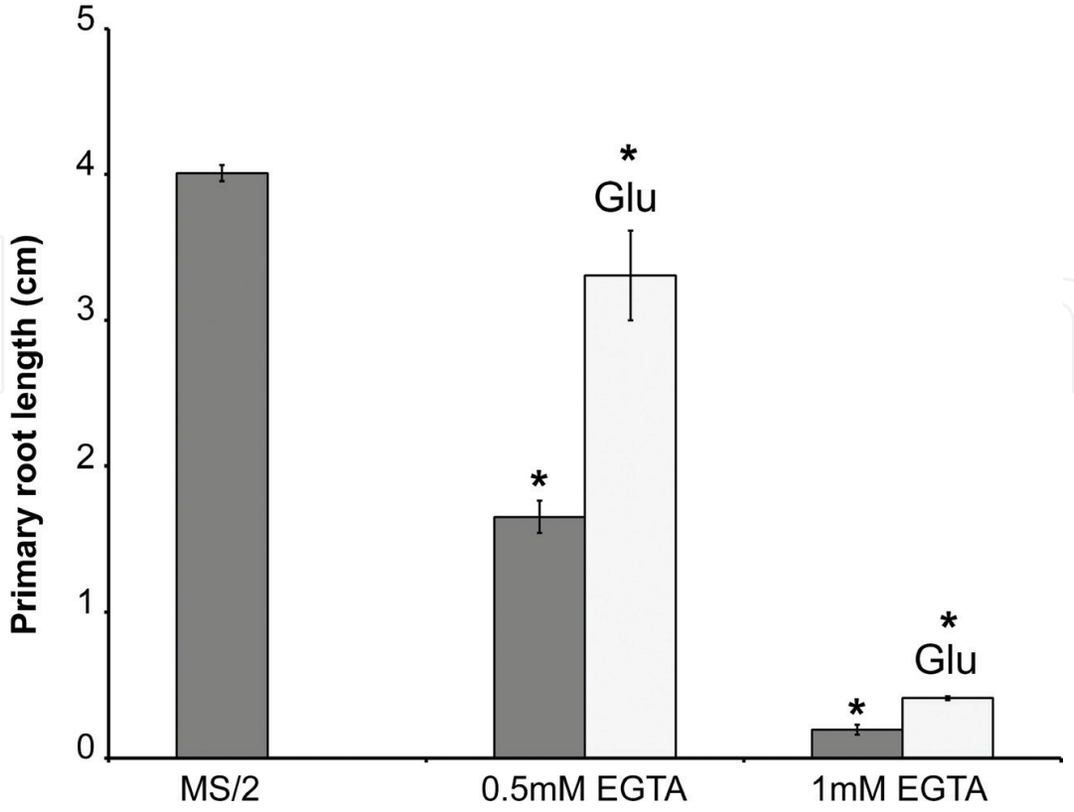

Figure 4. Ca^{2+}-dependent growth phenotypes of EGTA treated wild-type root seedlings. Putative ligand Glu can overcome reduced root growth by low amount of EGTA. Root phenotype of the 10-day-old wild-type seedlings under the different treatments. Supplement of 0.5 mM Glu successfully recovered the primary root growth which was reduced by 0.5 mM and 1 mM EGTA. The data presented are averages of three biological replicates. Asterisks represent statistical difference analyzed with a Student's *t*-test; $P < 0.005$, $n = 15$.

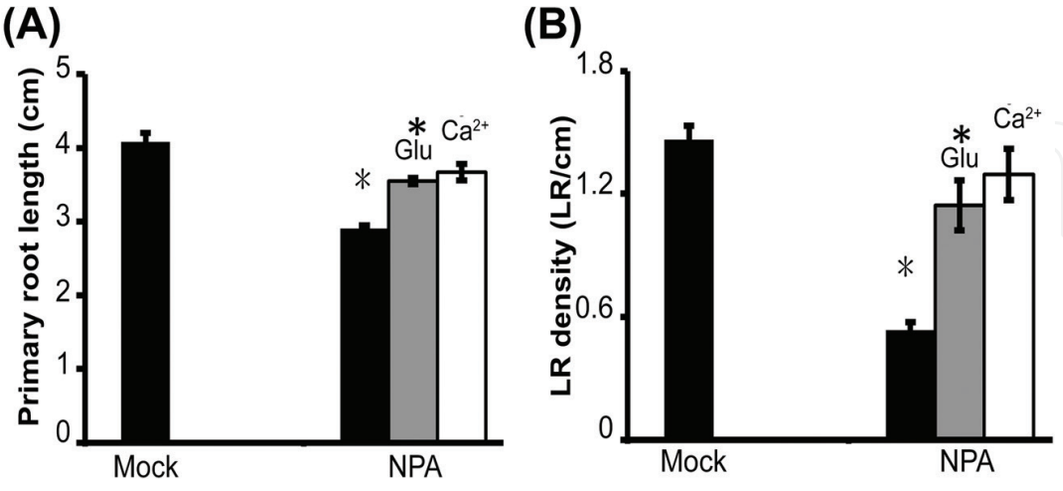

Figure 5. Reduced root growth shown by NPA-treated wild-type roots can be rescued by the externally supplied Glu. Recovery of arrested root growth suggest the role of auxin. Application 1-N-Naphthylphthalamic acid (NPA) caused arrest of root growth. However, exogenous application of Glu and Ca^{2+} (0.5 mM) to NPA-treated root is successfully minimized the NPA effect (A and B). Asterisks represent statistical difference analyzed with a Student's *t*-test; $P < 0.005$, $n = 15$.

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4. Discussion

4.1. Effect of glutamate treatment on root growth

Root plays pivotal role in plant life as it is crucial for nutrient and water absorption. In *Arabidopsis thaliana*, a total of 20 types of AtGLR subunits have been identified. They have significantly high sequence similarity with animal iGluR-like channels [46]. Probably due to phylogenetically conserved amino acid sequences, they may have a high potential for functional redundancy. Using specific drugs that alter the channel activities is a key to study the function of iGluR-like channels in Arabidopsis [21]. We used Glu, (a neurotransmitter), and other set of compounds known to agonize (activate) (NMDA and AMAP) and antagonize (deactivate) (DNQX and CNQX) the iGluR channels in mammalian cells. The use of broader set of drugs would allowed us to observe the specific effects related to Glu and iGluR-like channels in root cells. We observed that the application of these drugs potentially modulate the Arabidopsis root architectures indicating an importance for AtGLRs in root development. We observed that the application of iGluR agonists, Glu and NMDA were promoting root growth. In other studies it has also been reported that Glu could act as a root growth modifier [32, 47, 48]. Because Glu is an acidic amino acid which can cause low pH-induced toxicity (acidic), which could reduce root growth [31, 32, 48], we performed all experiments on constant pH range from 5.7 to 5.8 designed for plant tissue culture medium. Our result showing correlation with other evidences which have been proved that plants possess Glu-activated ion channels like iGluRs [30, 35, 36]. More specifically, NMDA-like iGluR receptors are also predicted in plants [27, 49]. Ammonium ion is a key form of inorganic nitrogen. Organic nitrogen compounds (amino acids, nucleic acids *etc*.) are derived from NH_4^+ [50]. The assimilation of NH_4^+ into Glu is the crucial step in amino acid synthesis and nitrogen metabolism [51]. Glu is directly involved chlorophyll synthesis in developing leaves [52]. Although it cannot be ruled out that Glu metabolism plays an important part in plant nitrogen assimilation and its regulation, increasing evidence suggests signaling properties of Glu in animals may also develop in plant [53].

The specificity of Glu to promote root development is individual. We used another kind of amino acid neurotransmitter, aspartate (Asp). Unlike Glu, it failed to induce root growth, showing Glu signaling in root development is highly specific [19]. Both DNQX and CNQX are the potent competitive AMPA/kainate glutamate receptor antagonists [38, 54]. We reported that iGluR antagonists have drastic effect on root growth. In animals they are known to block the ionotropic glutamate receptors very precisely [55]. Moreover, some studies in plants also have defined that animal iGluR antagonist are capable of changing the ion activity inside the cells and hence the phenotypes [16, 19, 21, 35, 56]. More interestingly, additional supply of Glu is able to counter the negative effect of each antagonist, suggesting a strong evidence of the existence of functional glutamate receptors in plant root development [17, 19, 20, 37, 56]. Similar evidence is also reported. Glu and Gly successfully revert back the effect of DNQX on Arabidopsis hypocotyl growth [27].

4.2. Root meristematic activity

In Arabidopsis, root meristem develops from a stem-cell niche situated at the apical part of the root [57, 58]. Glutamate Receptor–Like protein (GLR3.1) has been described to be

essential for meristematic activity in roots [22]. The roots grown by antagonist treatment significantly reduced meristematic cell number, and hence a contraction of meristem size was also observed. These observations certainly showed a correlation with less root growth under antagonist treatment [59]. The role of quiescent center (QC) is vital in the maintenance of root meristem [58, 60]. The majority of cells in the root meristem develop from stem cells which are derived from QC. In confocal microscopic analysis, antagonist treated-root showed a major change in QC organization which may resulted in less developed root meristem [61]. Numerous sedimented starch-filled amyloplasts in the root cap are distinguishing of columella cells [62]. In our study it was observed that columella cells of antagonist treated-roots possessed of defective amyloplasts in Lugol staining [63]. Therefore short root phenotype is highly consistence with defected organization of the root cap and QC [42, 64, 65].

4.3. Glutamate and calcium in root growth

The iGluR is known to be a Ca^{2+} permeable channel [66]. Many studies revealed that Arabidopsis AtGLR induces Ca^{2+} current upon activation by Glu [27, 30, 67]. We investigated whether the putative agonist and antagonist treatments alter the $[Ca^{2+}]$cyt level in roots. EGTA is a well-known Ca^{2+}-chelating agents [68]. In our study, application of EGTA shows a strong inhibition in root growth. Interestingly, however when Glu was introduced in same media, root growth was resumed. The presence of EGTA allows low availability of Ca^{2+} in free space. Animal cells and plant cells are similar in that they are both use endoplasmic reticulum (ER) as a calcium storage. Glutamate receptors are also reported to localized in ER [22, 69]. In animals, Glu-induced intracellular calcium levels through endoplasmic reticulum is reported [69]. However, application of Glu may lead to more activation of putative AtGLRs that allow more Ca^{2+} release to cytoplasm from endomembrane system which might play a role to recover the root growth. Calcium is key regulator of root growth [70, 71]. Previous report has also found that roots in EGTA containing media failed to grow toward gravity but it could be recovered by extra Ca^{2+} supply [72]. Furthermore, as we have discussed before that application of DNQX and CNQX reduced root apical meristem and hence also root growth, but application external Ca^{2+} could resume root growth. These results suggest a role of AtGLRs in Arabidopsis root development.

4.4. Glutamate signaling and polar auxin transport in roots

Expressions of *AtGLR* genes inside the root tissue give strong evidence that these receptors have vital role [46, 73]. Recent studies on chimeric and other plant iGLRs provided evidence for Ca^{2+} permeability across membranes. We have also found that the *glr3.6-1* mutant showed altered cytosolic calcium levels in root cells [24]. Calcium and auxin work together in many aspects of cellular processes. A similar effect has been observed in different studies in response to calcium-chelating agents. Dela Fuente and Leopold (1973) showed that basipetal transport of auxin is depressed by EDTA treatment and that subsequent addition of Ca^{2+} restores auxin transport in roots [74]. Root bend toward a calcium-containing agar block

versus an agar block with the calcium-chelating agent EGTA, suggesting that auxin transport is regulated by local $[Ca^{2+}]_{cyt}$ levels [72]. NPA is a potent polar auxin transport inhibitor, which can highly reduce the lateral root emergence [75–77]. Supplement of Glu together with NPA (1-N-Naphthylphthalamic acid) (at 0.5 mM) showed approximately close root phenotype to the control seedlings. Addition of Glu in intact roots directly may induce Ca^{2+} which may lead to enhanced auxin transport and hence the suppressed negative effect of NPA Possibly application of Glu can enhance the auxin supply to other deserved root cells rather than showing competition with NPA blockage.

5. Conclusion

In this study, we applied a comprehensive set of compounds to study how these compounds affect Arabidopsis root growth. Arabidopsis root system is highly sensitive to these compounds known to alter the iGluR channels. Both Glu and NMDA promote the primary root growth and lateral root density in Arabidopsis. On the other hand, iGluR antagonists drastically reduced root growth at both parameters. Exogenous application of Glu successfully rescued reduced root phenotype inhibited by EGTA. Moreover, root growth reduced by polar auxin transport inhibitor NPA, could be rescued by Glu and $CaCl_2$. As for AtGLRs function, although the mechanisms are not yet clear, the results presented provide evidence in support of a role of AtGLRs in regulating Arabidopsis root development.

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