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The Role of Glutamate Receptor *AtGLR3.7* and Mutants in *Arabidopsis thaliana* Growth

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Abstract

Glutamate receptor-like genes (GLRs) in Arabidopsis thaliana have been implicated in various physiological processes, yet the specific role of AtGLR3.7 remains poorly understood. In this study, we investigated the impact of altered AtGLR3.7 expression on plant development by analysing three overexpression lines and one knockout line, all in a Col-0 background. The overexpression lines were selected to represent a gradient of AtGLR3.7 expression levels. Phenotypic analyses revealed that AtGLR3.7 positively regulates seed size, root length and aerial tissue expansion in a dose-dependent manner. Overexpression lines exhibited enlarged seeds and accelerated rosette and stem growth, while the knockout line showed reduced development in all traits. Root architecture remained largely unchanged across genotypes, except for a reduced primary root length in the knockout, which led to a higher lateral root density. Exposure to exogenous L-glutamate revealed that overexpression of AtGLR3.7 increases plant sensitivity to glutamate-induced root growth inhibition, whereas the knockout line displayed an unexpected stimulation of root growth under high glutamate concentrations. Interestingly, 250 µM L-glutamate was sufficient to restore normal root growth in both mutant backgrounds, suggesting a critical balance between AtGLR3.7 expression and glutamate availability. These findings support a model in which AtGLR3.7 modulates root meristem activity through glutamate signaling, potentially by influencing auxin dynamics at the root tip. Our results highlight AtGLR3.7 as a key modulator of plant growth and glutamate responsiveness and provide new insights into the functional specificity of GLRs in Arabidopsis development.

Keywords: Glutamate receptor, knockout line, Overexpression lines, Root architecture.

1. Introduction

A living being, whether animal or plant, needs information exchange between its subunits (cells) because when receiving external stimuli in one part of its body, the organism can show responses in another part. In humans, cell-cell communication can occur in different ways. One form of communication between cells can be through the nervous system, more precisely through neurons. These interactions are called synapses, which usually occur between the axon termination of the emitting cell (presynaptic neuron) and the dendrites of the receptor neuron (postsynaptic neuron) [1]. There are two types of synapses: the electric, based on an electrical pulse transmitted from an axon to a dendrite; and chemical, also initiated by an electrical impulse but mediated by neurotransmitters. These neurotransmitters are chemical compounds that regulate cell-cell information exchange, such as ATP, acetylcholine, γ-aminobutyric acid (GABA) and glutamate [1].

Glutamate is a simple, excitatory neurotransmitter derived from an amino acid sequence [1]. Following successive stimuli in humans, glutamate is released by the presynaptic neuron, inducing the opening of ionotropic glutamate receptor channels (iGluR) in the postsynaptic neuron. This opening of iGluR allows glutamate entrance into the postsynaptic neuron and also allows calcium (Ca^{2+}) transport, because iGluRs are integral transmembrane proteins allowing calcium efflux (Ca^{2+}), causing membrane depolarization [2].

Amino acid signaling is not a unique feature of humans. Glutamate like receptor (GLR) and their proteins can be found in many bacteria, invertebrate animals, vertebrate animals, and even plants [3]. In 1998, plant genes similar to iGluR were discovered in the Arabidopsis thaliana genome [4]. This plant protein sequences had a strong structural resemblance to these animal glutamate receptors that are involved in fast synaptic responses in neuronal structures.

In animals, these receptors participate in a broad spectrum of neuronal processes such as memory and learning and are also implicated in neurodegenerative conditions like Alzheimer's and Parkinson's disease [5].

Since their discovery, GLRs have been found to be expressed in all parts of the plant, indicating their likely role as universal sensors and mediators of a wide range of endogenous and exogenous signals [6]. The GLR system appears to be highly regulated based on gene expression analysis under internal or external stress applications [7].

GLRs can be found in various plant cell types, not only in A. thaliana but probably in all plant species [6], as has been reported in rice [8], radish [9], grass [10] and liverworts [11]. In mammals, iGluR play key roles in central nervous system functioning [12] in cognition, learning, and memory [1, 13]. In plants, GLRs have several functions such as regulation of hypocotyl elongation [4, 14], abscisic acid (ABA) synthesis [15], regulation of root meristem [8, 16], plant defence against pathogens [17], long-range wound mediated signaling calcium (Ca^{2+}) [18, 19] and stomatal closure modulation through Ca^{2+} [20].

The 20 types of glutamate receptors found in A. thaliana (AtGLR) are distributed into three clades according to similarity in DNA sequence [21]. Clade I genes are more expressed in shoots, clade II genes are more expressed in the roots, and clade III genes can be expressed in the entire plant [21-23]. Because they are expressed throughout the plant, clade III genes have been widely studied, especially for their action on calcium signaling [6]. AtGLR3.1, -3.2, -3.6 and -3.7 probably enter the secretory pathway and are incorporated into the PM while the others (AtGLR3.3, -3.4 and -3.5) contain sequences for multiple cellular targets [24, 25]. GFP-tagged versions of AtGLR3.2, -3.3 and -3.4 have been found scattered among all cell types within the root growth zone whereas a concentration at the PM of sieve plates within the phloem of the mature region of the root was observed [21, 26]. The accumulation at the junction of phloem elements appears to be connected with the involvement of GLRs in long-distance wounding signal transduction as it is seen during an herbivore attack [18].

One of the 20 types of AtGLR found in A. thaliana that has not been extensively studied is the AtGLR3.7. In this we studied the phenotypes of Glutamate Receptor AtGLR3.7 and its mutants and the roles this gene play in the development of plant. Recently a comparison between differences in root skototropic behaviour across different expression lines of Arabidopsis thaliana seedlings (atglr3.7 ko, AtGLR3.7 OE, and pin2 knockout) were made to comprehend their functions. This research shows that as the distance between roots and darkness increases, the root's positive skototropism noticeably weakens. Hence highlight the involvement of GLR3.7 and PIN2 in root skototropism [27]. Gong et al 2025 reported on reduced sensitivity of root growth to L-Glu of all 20AtGLR mutants' genes screened. The aim of this study is to investigate the role of the glutamate receptor AtGLR3.7 and its mutant lines in Arabidopsis thaliana, with a focus on their impact on root development and responsiveness to glutamate, contributing to the broader understanding of GLR-mediated signaling in plants.

2. Material and Methods

Transformation of *Arabidopsis thaliana* (Col-0) via Floral Dip

The pBI-AtGLR3.7 and pMDC7-AtGLR3.7 vectors in *Agrobacterium tumefaciens* GV3101 were transformed into *Arabidopsis thaliana* (Col-0) using the floral dip method by Clough and Bent (1998). Plants at the flowering stage were dipped into a solution of Agrobacteria harbouring the respective vector. Collected seeds were surface sterilized and selected on Murashige-Skoog (MS) agar plates containing kanamycin (50 µg mL-1; plant selection for pBI-AtGLR3.7) or hygromycin B (10 µg mL-1; plant selection for pMDC7-AtGLR3.7). Homozygous plants harbouring either pBI-AtGLR3.7 (designated OE:AtGLR3.7(1), -(2) and -(3)) or pMDC7-AtGLR3.7 (designated I:AtGLR3.7(1) and -(2)) were obtained in the T3 generation using segregation assays. MS medium's pH was adjusted to 5.7 before autoclaving for 20 min at 121°C, 103.421 kPa. Antibiotics were added when the medium had cooled down to about 60°C after autoclaving. Phytagel (4 g) was added before autoclaving when solid medium was required.

All plant genotypes used in this study had the background of *Arabidopsis thaliana* Col-0. The *AtGLR3.7* knockout line, atglr3.7, was kindly provided by Prof. Lai-Hua Liu (China Agriculture University Beijing). Different plant genotypes and growth conditions were used depending on the experiment. If not explicitly mentioned, the standard growth conditions that followed were applied. Sterile growth conditions were maintained by surface sterilization of Arabidopsis seeds. Rough sterilization was done in 70% ethanol for 1 min, followed by a thorough sterilization in a sterilization solution for 10 min. Seeds were washed at least five times in sterile ddH2O.Sterilized seeds were either (I) plated on sterile filter paper in a petri dish and stored at 4°C in darkness, (II) plated directly on sterile ½ MS-agar medium in round (90 mm) or square-shaped (120x120 mm) petri dishes or (III) put in liquid, sterile MS-medium for hydroponic growth. Plants were always stratified for 3 days at 4°C in darkness before they were transferred to controlled growth chambers (temperature: 22°C; light intensity: 110 mmol m-2 s-1; photoperiod: 16 h light/8 h dark; humidity: 70%). The plant age was calculated based on seed imbibition and therefore included the stratification period of 3 days (DAI: days after imbibition). Individual plants, or parts of them were digitalized with either a scanner or a camera. Analysis of the obtained images were done with the respective manufacturer software. Additional image analysis was conducted with the free, open-source software 'ImageJ' (https://imagej.nih.gov/ij/) and 'GIMP' (https://www.gimp.org/).

Arabidopsis seeds were sown on square-shaped ½ MS agar plates. After stratification, petri dishes were wrapped in aluminium foil and transferred to growth chambers. Seedlings were grown in a vertical position for ten days. At the end of the experiment, plates were digitalized with a scanner and hypocotyl elongation as well as primary root length measured with 'ImageJ' software

Root growth measurements were done using sterilized Arabidopsis seeds sown on ½ MS agar in petri dishes. After stratification, plants were transferred to a growth chamber and root growth was marked with a pen on the petri

dish in distinct time periods ranging from one to three days. At the end of the desired growth period, petri dishes were digitalized using a scanner and evaluated using 'ImageJ' and 'GIMP' image-analysing software.

The inducible expression of *AtGLR3.7* in I:AtGLR3.7(1) and I:AtGLR3.7(2) was initiated by growing these plants and Col-0 as a control on ½ MS agar containing 5 µM 17-β-estradiol. The effect of glutamate on plant growth was observed by growing Col-0, atglr3.7 and OE:AtGLR3.7(2) on ½ MS agar containing 50, 250 or 1,000 µM L-glutamate (D-glutamate served as a negative control). Mock treatments were performed using standard ½ MS agar.

Plants were grown in soil under standard conditions. Pictures with a reference marker were taken with a camera. Photographing continued daily for six days after the rosette reached its final size, about 35 DAI. Image analysis was performed with 'ImageJ' software. Stem growth was measured with a flexible ruler starting about 23 DAI when the inflorescence stems emerged. The number of side bolts and branches on the main stem were counted by eye. All measurements were recorded at intervals of every two to three days.

3. Results

An investigation of the different plant phenotypes was done by observing the seeds of Col-0 and the transgenic plant lines atglr3.7, OE:AtGLR3.7(1), OE:AtGLR3.7(2) and OE:AtGLR3.7(3). An increase in total seed size was noted for all *AtGLR3.7* overexpression lines whereas the knockout line exhibited a slightly reduced seed size (Figure 1). More in-depth measurements revealed highly significant variations among all plant lines in the four tested parameters (seed area, perimeter, major and minor axis) (one-way ANOVA, $\rho < 0.0001$, n = 68-108). The overexpression lines were generally characterized by an increase in the observed seed characteristics when compared to Col-0 (

Table-1).

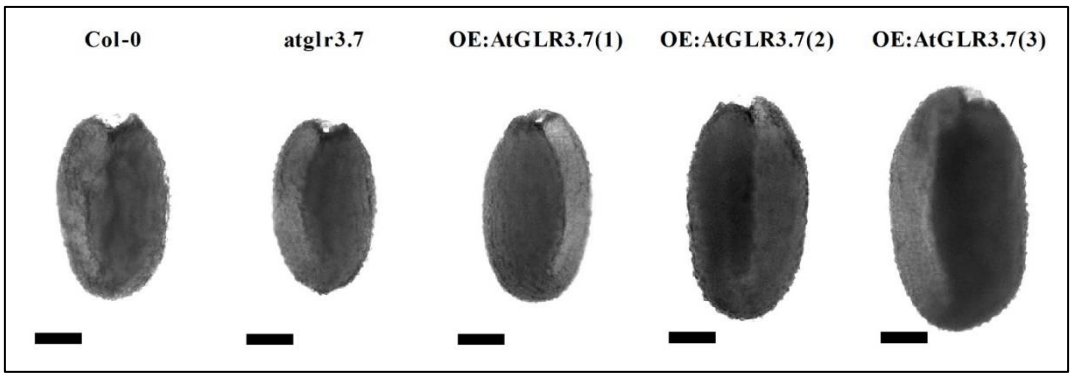

Figure-1. Morphological Comparison of Seeds. Seeds of Col-0, atglr3.7, OE:AtGLR3.7(1), OE:AtGLR3.7(2) and OE:AtGLR3.7(3) serving as examples for the respective *Arabidopsis thaliana* line. Siliques containing the displayed seeds were harvested from ten-week-old plants. Black bars represent 100 µm.

Table-1. Detailed Characteristics of Seeds from Transgenic and Wildtype *Arabidopsis thaliana*.

Plant line	Seed area (mm^2 ± SD)	Seed perimeter (mm ± SD)	Seed major axis (mm ± SD)	Seed minor axis (mm ± SD)
Col-0	0.108 ± 0.014	1.47 ± 0.12	0.502 ± 0.033	0.269 ± 0.022
atglr3.7	0.103 ± 0.014	1.36 ± 0.11 (***)	0.471 ± 0.035 (***)	0.281 ± 0.021 (**)
OE:AtGLR3.7(1)	0.115 ± 0.016 (*)	1.43 ± 0.12	0.491 ± 0.040	0.294 ± 0.022 (***)
OE:AtGLR3.7(2)	0.133 ± 0.016 (***)	1.56 ± 0.12 (***)	0.542 ± 0.032 (***)	0.314 ± 0.022 (***)
OE:AtGLR3.7(3)	0.149 ± 0.027 (***)	1.64 ± 0.17 (***)	0.568 ± 0.056 (***)	0.324 ± 0.030 (***)

Statistical analyses were conducted using a one-way ANOVA and Dunnett's *post hoc* test, n = 68-108; *$\rho < 0.05$, **$\rho < 0.01$ and ***$\rho < 0.001$.

The development of the root system on 14 DAI differed visually among the tested genotypes (Figure 2). Continuous measurements over a period of 14 days allowed for a development-dependent characterisation of the plant's primary root length and its daily root growth. The root length of OE:AtGLR3.7(2) and -(3) showed a constant surplus compared to Col-0 while the root of atglr3.7 continued to be shorter than in wildtype plants (Figure 3A). A look on the daily root growth rate showed a significant, additional growth up to 41% on 7 DAI for OE:AtGLR3.7(3) compared to Col-0 (Figure 3B). This boost in root growth abated down to 30% on 9 DAI and only 6% on 13 DAI. The knockout of *AtGLR3.7* led to a constantly diminished daily growth compared to Col-0 down to -33% on 7 DAI and about -15% during later growth stages.

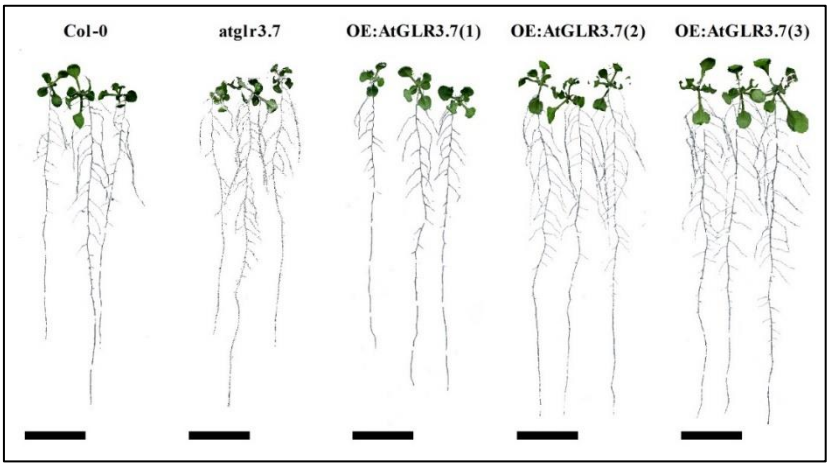

Figure-2. Developed Root System on 14 DAI. Seedlings serving as examples for the respective Arabidopsis plant line. Wildtype and transgenic *Arabidopsis thaliana* were grown in ½ MS agar for two weeks. Black bars represent 20 mm.

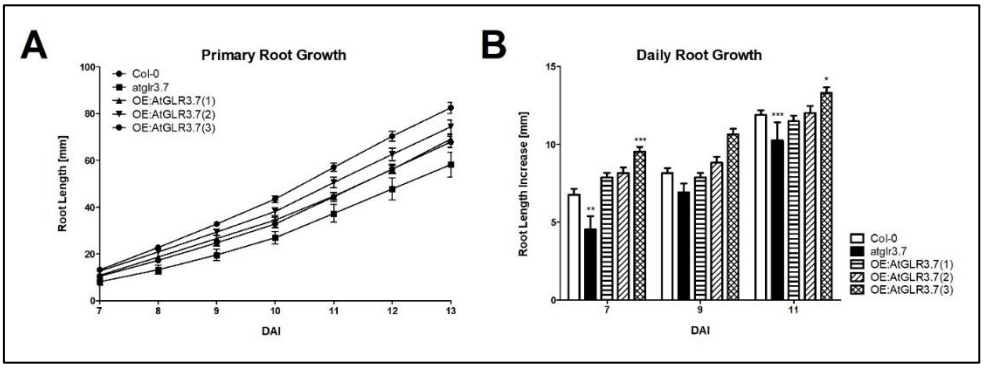

Figure-3. Primary Root Length and Daily Root Growth. Primary root length (A) and daily root growth (B) of wildtype and transgenic *Arabidopsis thaliana* grown on ½ MS agar were measured regularly for two weeks. Daily measurements started from 7 DAI onwards. The *AtGLR3.7* overexpression lines showed an increase in primary root length whereas atglr3.7 was characterized by a reduction in primary root development. Statistical analyses among genotypes were conducted using a one-way ANOVA and Dunnett's *post hoc* test; n = 11-19. Asterisks indicate significant deviations from Col-0 with *$\rho < 0.05$, **$\rho < 0.01$ and ***$\rho < 0.001$. Error bars indicate SE.

An investigation of the root architecture showed no significant quantitative or qualitative deviations in lateral root development among the tested plant lines (

Table-2). However, atglr3.7 exhibited a slight increase in secondary root density of about 11.65% while its final primary root length showed a reduction of -14.20% compared to Col-0. OE:AtGLR3.7(3) that had a significantly higher-developed root architecture than wildtype plants with an increase of 17.96% in primary root length and 11.53% in root diameter. OE:AtGLR3.7(2) was also characterized by a longer primary root (9.65%) and a significantly wider root diameter (8.23%). OE:AtGLR3.7(1) showed a root phenotype similar to Col-0. Determinations of the beginning of the root hair zone showed no deviations among the plant lines. First root hairs emerged at a distance of 1.25 ± 0.02 mm from the root tip in all tested Arabidopsis genotypes.

Table-2. Root Architecture of Transgenic and Wildtype *Arabidopsis thaliana* on 14 DAI.

Plant line	**1° root length (mm ± SD)**	**1° root diameter (µm ± SD)**	**Start of root hair zone (mm ± SD)**	**2° roots per cm (# ± SD)**
Col-0	67.88 ± 10.04	154.4 ± 6.4	1.23 ± 0.09	3.69 ± 0.82
atglr3.7	58.24 ± 17.76	153.5 ± 9.8	1.27 ± 0.14	4.12 ± 1.32
OE:AtGLR3.7(1)	69.18 ± 5.49	156.7 ± 5.6	1.27 ± 0.04	3.60 ± 0.25
OE:AtGLR3.7(2)	74.43 ± 12.17	167.1 ± 12.9 (*)	1.25 ± 0.13	3.73 ± 0.66
OE:AtGLR3.7(3)	80.07 ± 7.69 (*)	172.2 ± 5.8 (**)	1.23 ± 0.09	3.73 ± 0.42

Statistical analyses were conducted using a one-way ANOVA and Dunnett's *post hoc* test, n = 9-19; *$\rho < 0.05$, **$\rho < 0.01$.

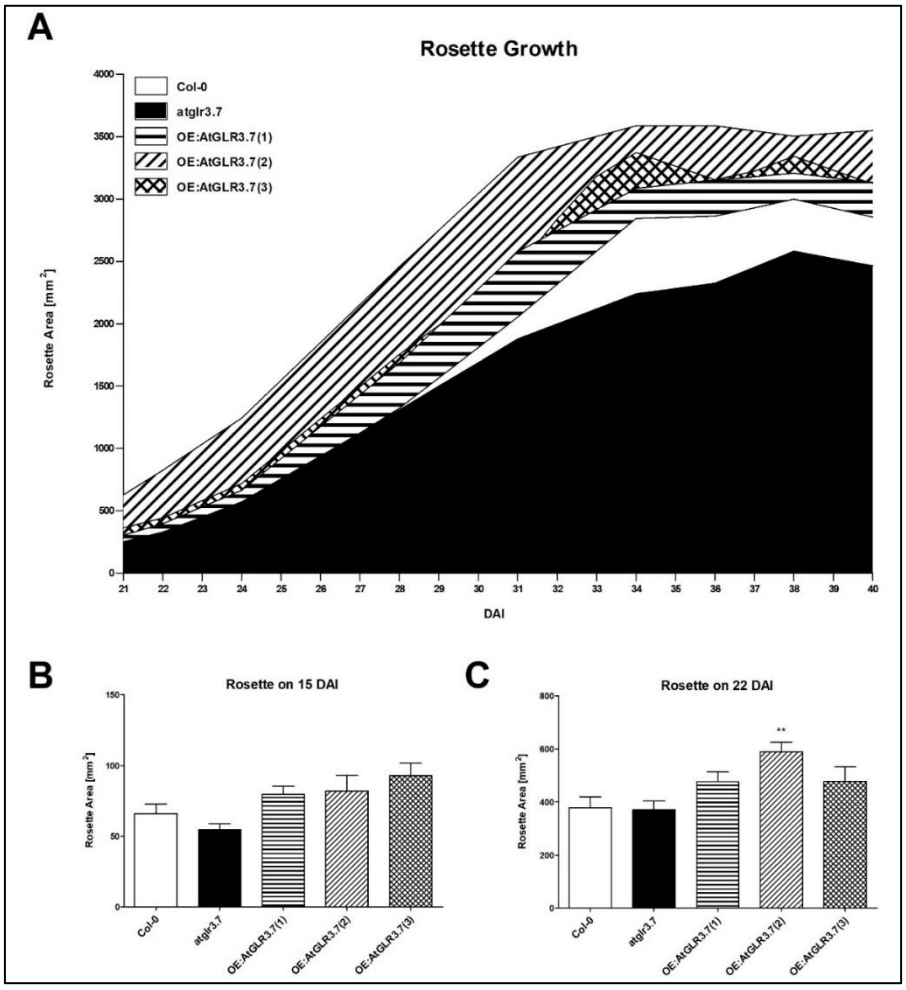

Figure-4. Rosette Growth and Size at Different Developmental Stages. Rosette area of wildtype and transgenic *Arabidopsis thaliana* grown in soil was measured daily during a plant life cycle until 40 DAI (A). Rosette size of plants grown on ½ MS agar on 15 DAI (B) and plants grown in soil at the beginning of the reproductive stage (C) serve as reference marker for specific plant development stages. The *AtGLR3.7* overexpression lines were characterized by a tendency of increased rosette growth which was especially prominent in OE:AtGLR3.7(2). Statistical analyses among genotypes were conducted using a one-way ANOVA and Dunnett's *post hoc* test; n = 5-18. Asterisks indicate significant deviations from Col-0 with $^{**}\rho < 0.01$. Error bars indicate SE.

An investigation of the rosette growth revealed variations among transgenic and wildtype plants grown in soil (Figure 4A). Following the rosette development over a period of 40 d, atglr3.7 showed the least rosette growth with a decrease in rosette size of -14% compared to Col-0. OE:AtGLR3.7(1) and OE:AtGLR3.7(2) showed an overall increase in their rosette size of 11 and 28%, respectively. The deviations were especially prominent in early plant development stages as it can be seen for plants grown in petri dishes on 15 DAI (Figure 4B). At this stage, OE:AtGLR3.7(3) grew bigger than OE:AtGLR3.7(2) while atglr3.7 exhibited clearly a smaller rosette. When reaching the reproductive stage, OE:AtGLR3.7(2) showed a significant increase of about 56% in rosette area compared to Col-0 whereas OE:AtGLR3.7(3) exhibited only a minor increase similar to OE:AtGLR3.7(1) in comparison to wildtype plants (Figure 4C).

A detailed comparison of rosette parameters revealed a constant and significant increase in rosette area and expansion (major and minor radius) only for OE:AtGLR3.7(2) (Table-3). OE:AtGLR3.7(1) and OE:AtGLR3.7(3) also showed increases in their rosette dimensions but to a lesser extent. Furthermore, the initial growth boost in these latter plant lines was less stable throughout the plant's life cycle (Figure 4A).

Table-3. Rosette Characteristics of Transgenic and Wildtype *Arabidopsis thaliana* Grown in Soil on 22 DAI.

Plant line	Rosette area (mm^2 ± SD)	Rosette perimeter (mm ± SD)	Rosette major radius (mm ± SD)	Rosette minor radius (mm ± SD)
Col-0	378.04 ± 92.22	250.72 ± 46.13	24.28 ± 2.83	19.53 ± 2.81
atglr3.7	371.38 ± 72.83	251.53 ± 43.45	23.49 ± 1.85	19.95 ± 2.46
OE:AtGLR3.7(1)	420.00 ± 126.76	266.70 ± 71.82	25.57 ± 2.77	20.48 ± 4.61
OE:AtGLR3.7(2)	588.80 ± 80.86 (**)	334.96 ± 60.79	29.11 ± 1.54 (*)	25.65 ± 2.33 (*)
OE:AtGLR3.7(3)	477.47 ± 109.39	259.76 ± 56.03	25.93 ± 3.69	23.14 ± 2.19

Statistical analyses were conducted using a one-way ANOVA and Dunnett's *post hoc* test, n = 5-6; *ρ < 0.05, **ρ < 0.01.

Starting from the onset of the reproductive stage of *Arabidopsis thaliana*, the shoot transforms into an inflorescence stem while side bolts emerge from the rosette. An investigation of the stem in transgenic and wildtype Arabidopsis plants showed a significant and constant increase in growth for OE:AtGLR3.7(2) (Figure 5). OE:AtGLR3.7(1) and OE:AtGLR3.7(3) exhibited an initial boost in stem growth similar to OE:AtGLR3.7(2) until 31 DAI. However, this growth acceleration abated until it was comparable to Col-0 and atglr3.7 during the final stages of stem growth/development.

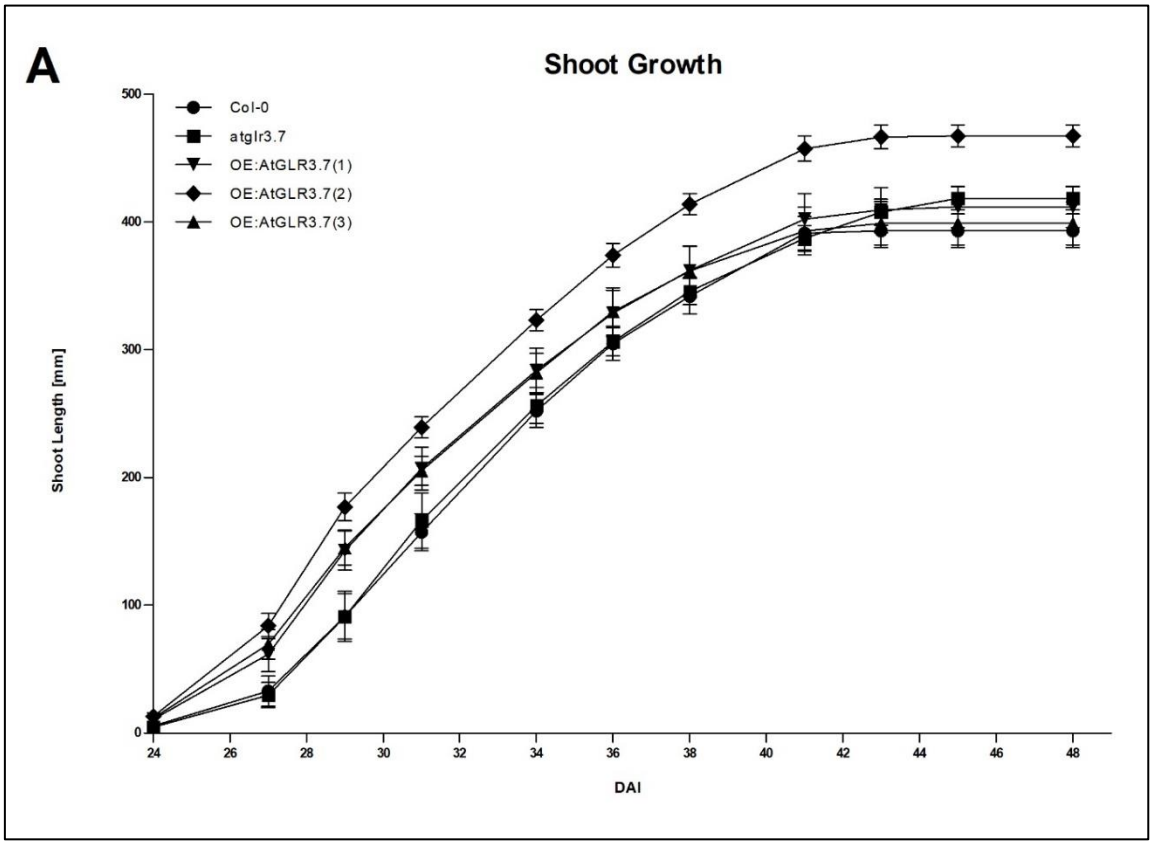

Figure-5. Shoot Growth During the Reproductive Stage. Shoot length of wildtype and transgenic *Arabidopsis thaliana* grown in soil was measured every two to three days. All *AtGLR3.7* overexpression lines showed an initial boost in stem growth while only OE:AtGLR3.7(2) exhibited a constant growth acceleration. OE:AtGLR3.7(1) and -(3) displayed a reduction in daily stem growth starting from 34 DAI. The *AtGLR3.7* knockout line was characterized by a stem growth comparable to Col-0. Error bars indicate SE, n = 5-6.

The morphology of the stem including the side branches was similar among all tested plant lines that were characterized by 2.8 ± 0.2 branches on the main stem (Table 4). The number of side bolts emerging from the rosette increased about 21 and 9% in OE:AtGLR3.7(2) and OE:AtGLR3.7(3), respectively. The *AtGLR3.7* knockout line showed a minor reduction of about -12% compared to Col-0 while OE:AtGLR3.7(1) exhibited no deviations from wildtype plants. OE:AtGLR3.7(2) was the only transgenic plant line which showed a strong increase in final stem length of about 19% compared to Col-0.

Table-4. Stem and Side Bolt Growth in Transgenic and Wildtype *Arabidopsis thaliana* on 48 DAI.

Plant line	Final stem length (mm ± SD)	Branches on main stem (# ± SD)	Side bolts (# ± SD)
Col-0	404.60 ± 16.50	3.0 ± 0.6	5.7 ± 1.0
atglr3.7	418.30 ± 21.92	3.0 ± 0.6	5.0 ± 0.6
OE:AtGLR3.7(1)	411.60 ± 35.93	2.6 ± 0.5	5.8 ± 1.3
OE:AtGLR3.7(2)	467.20 ± 20.89 (**)	2.7 ± 0.5	6.6 ± 0.9
OE:AtGLR3.7(3)	398.80 ± 37.80	2.6 ± 0.5	6.2 ± 1.1

Statistical analyses were conducted using a one-way ANOVA and Dunnett's *post hoc* test, n = 5-6; **$\rho < 0.01$.

Since hypocotyl growth in darkness is solely a process of cell elongation without cell division, the experimental setup was designed to reveal the role of cell elongation in the observed growth acceleration/decrease of the transgenic *Arabidopsis thaliana*. Plants were germinated and grown under sterile conditions in darkness for ten days, a condition that promoted strong hypocotyl elongation with no deviations in hypocotyl length among the tested plant lines (Figure 6).

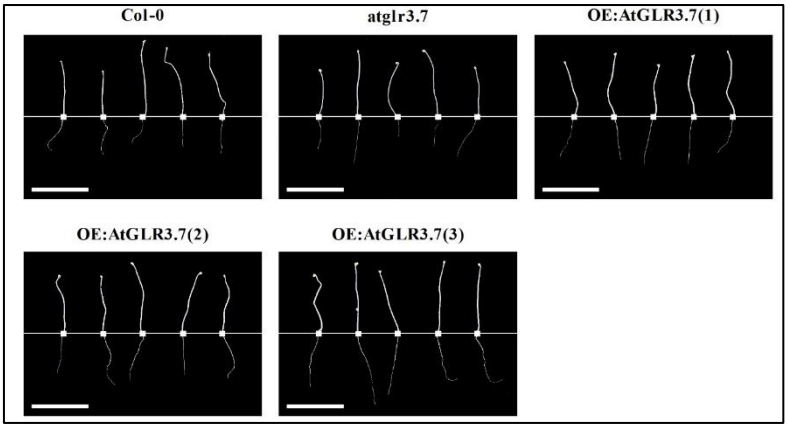

Figure-6. Growth of Seedlings in Darkness on 10 DAI. Wildtype and transgenic *Arabidopsis thaliana* were grown on ½ MS agar in petri dishes covered with aluminium foil under otherwise standard conditions. Seedlings were removed from petri dishes and digitalized on 10 DAI. White bars represent 20 mm.

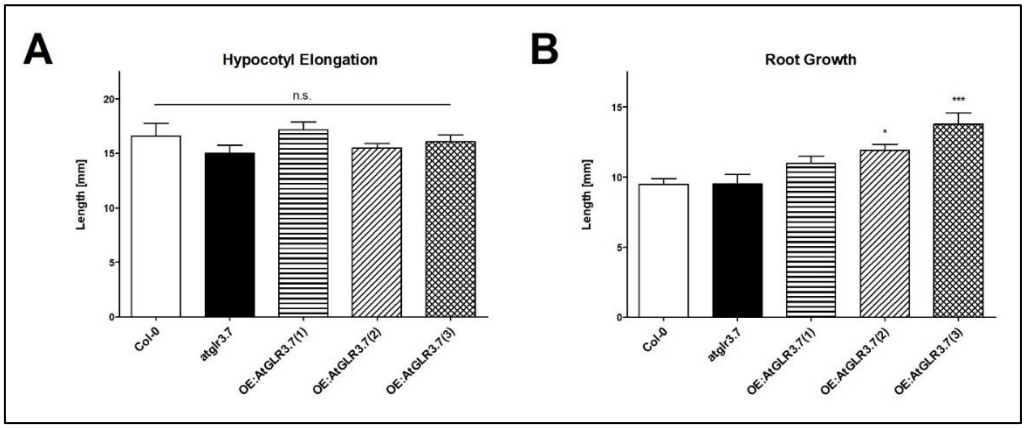

Figure-7. Hypocotyl Elongation and Primary Root Length in Darkness on 10 DAI. Wildtype and transgenic *Arabidopsis thaliana* were grown under sterile conditions on ½ MS agar in darkness. The hypocotyl elongation was comparable among all tested plant lines (A). Primary root length was significantly increased in the two *AtGLR3.7* overexpression lines with the highest *AtGLR3.7* expression levels (B). Measurements were done after removing the seedlings from the petri dishes wrapped in aluminium foil. Statistical analyses among genotypes were conducted using a one-way ANOVA and Dunnett's *post hoc* test; n = 16-20. Asterisks indicate significant deviations from Col-0 with *$\rho < 0.05$ and ***$\rho < 0.001$. Error bars indicate SE.

Comparisons among the different plant genotypes regarding their hypocotyl elongation showed no significant variations (Figure 7A). The average elongation rate was 16.04 ± 0.76 mm on 10 DAI. However, the primary root length showed significant differences when comparing among the overexpression lines with Col-0 (Figure 7B).

Here, the final root length of OE:AtGLR3.7(3) and OE:AtGLR3.7(2) was significantly higher with 13.78 ± 3.50 and 11.90 ± 1.90 mm compared to 9.48 ± 1.58 mm in Col-0, which equals an increase of 45 and 26%, respectively. OE:AtGLR3.7(1) exhibited an only slightly longer root (10.98 ± 2.18 mm) with an increase of about 16% compared to wildtype plants. The *AtGLR3.7* knockout line was characterized by an average root length of 9.51 ± 2.95 mm, similar to that of Col-0.

The effect of L-glutamate as a potential ligand and activator of GLRs was investigated at three different concentrations (50, 250 and 1,000 µM; and control) on *Arabidopsis thaliana* Col-0, atglr3.7 and OE:AtGLR3.7(2). Significant variations between the different plant lines were detected depending on the applied L-glutamate concentrations. A strong root growth reduction was observed at the highest concentration of 1,000 µM L-glutamate in OE:AtGLR3.7(2) whereas atglr3.7 experienced a notably boost in root growth . The experiments were also conducted with the enantiomer D-glutamate, which served as a negative control, but no major deviations between different concentrations or genotypes could be observed

Varying L-glutamate concentration had only minor effects on the final primary root length of Col-0 (Figure 8A). Comparing the final root length of the three different plant lines at the varying amino acid concentrations, a highly significant growth boost of OE:AtGLR3.7 against Col-0 during a mock treatment abated already at 50 µM L-glutamate and was no longer detected at higher concentrations. On the other hand, the *AtGLR3.7* knockout line was characterized by a highly significant increase in primary root length at 1,000 µM L-glutamate compared to the other plant lines (Figure 8B).

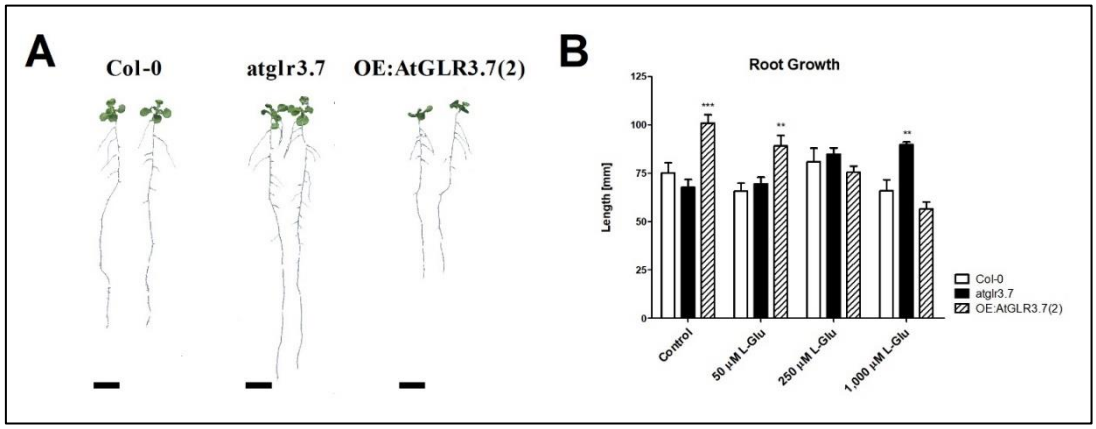

Figure-8. Effect of L-Glutamate on Root Development on 14 DAI. Wildtype and transgenic *Arabidopsis thaliana* were grown on ½ MS agar containing different L-glutamate concentrations (50, 250 and 1,000 µM). Plant growth in response to 1,000 µM L-glutamate (A). Primary root growth was reduced in an amino acid concentration-dependent manner in OE:AtGLR3.7(2) whereas atglr3.7 responded to increasing L-glutamate concentrations with an accelerated root growth (B). Statistical analyses among genotypes were conducted using a one-way ANOVA and Dunnett's *post hoc* test; n = 7-9. Asterisks indicate significant deviations from Col-0 with **$\rho < 0.01$. Error bars indicate SE. Black bars represent 10 mm.

The initial root length measured on plants before they were transferred to new, glutamate-containing plates on 7 DAI served as a reference marker for the relative root growth during the amino acid treatment. This relative root growth allowed an accurate estimation of the effect of L-glutamate on the plant root system (0). L-glutamate had minor effects on root growth in wildtype *Arabidopsis thaliana* (0A). Only a high concentration of 1,000 µM L-glutamate led to an insignificant reduction in primary root length in Col-0. The *AtGLR3.7* knockout line experienced a constant and significant boost in root growth at L-glutamate concentrations of 250 and 1,000 µM equalling an additional growth of about 19 and 26%, respectively (0C). The opposite effect was observed for OE:AtGLR3.7(2) in which increasing concentrations of L-glutamate caused continuing, highly significant root growth reductions especially at the highest amino acid concentrations of 250 and 1,000 µM (0E). Here, the primary root was about -25 and -44% shorter, respectively, than in the mock treatment.

An investigation of the daily root growth helps to illustrate the temporal process of an exposure to glutamate. The daily increase in root length fluctuated around 9.14 ± 1.79 mm in Col-0 with only minor deviations occurring at a L-glutamate concentration of 1,000 µM where plants responded with a significant reduction down to 7.35 ± 1.87 mm during advanced exposure periods on 13 and 14 DAI (Figure 9B). However, the daily root growth of 8.77 ± 1.42 mm in atglr3.7 during a mock treatment was significantly increased starting from a L-glutamate concentration of 250 µM on upwards and peaked with an average daily root growth of 13.32 ± 0.22 mm at a concentration of 1,000 µM L-glutamate (Figure 10D). Contrary, a highly significant decrease in daily root growth was observed in OE:AtGLR3.7(2) where an average growth of about 13.64 ± 1.20 mm during a mock treatment was diminished in an amino acid concentration-dependent manner down to 4.48 ± 1.41 mm at 1,000 µM L-glutamate (Figure 9F).

Comparing the root growth of Col-0, atglr3.7 and OE:AtGLR3.7(2) at particular L-glutamate concentrations, previous results concerning the root growth were reiterated in the mock treatment of all three plant lines where OE:AtGLR3.7(2) experienced a primary root growth boost while atglr3.7 showed a slightly reduced root growth compared to Col-0. Looking at the deviation in primary root growth in atglr3.7's and OE:AtGLR3.7(2) from

wildtype plants, it was shown that the overexpression line displayed a steady increase in root length of about 45% while the *AtGLR3.7* knockout line was characterized by an ongoing reduction in root length of about -5% compared to Col-0. A minor L-glutamate concentration of 50 µM reduced the additional root growth in OE:AtGLR3.7(2) slightly during early exposure periods where its root length was only about 32% longer than Col-0. The *AtGLR3.7* knockout line showed under the same conditions a minor but steady alleviation of its growth reductions (Figure 9 C, D). However, a concentration of 250 µM L-glutamate led to a highly significant reduction in primary root length in OE:AtGLR3.7(2) which caused a similar root length to Col-0 after one week of amino acid treatment (1E, F). A strong and highly significant increase in primary root length in atglr3.7 was caused by the same amino acid concentration leading to a root length likewise to Col-0 after one week (Figure 9 E, F). The highest tested concentration of 1,000 µM L-glutamate provoked a severe growth reduction on the *AtGLR3.7* overexpression line already early during the amino acid treatment which continued over the time course of the experiment while atglr3.7 matched the root length of Col-0 three days after the beginning of an L-glutamate application and excelled the primary root length of wildtype plants with an additional increase of about 35% at the end of the experiment.

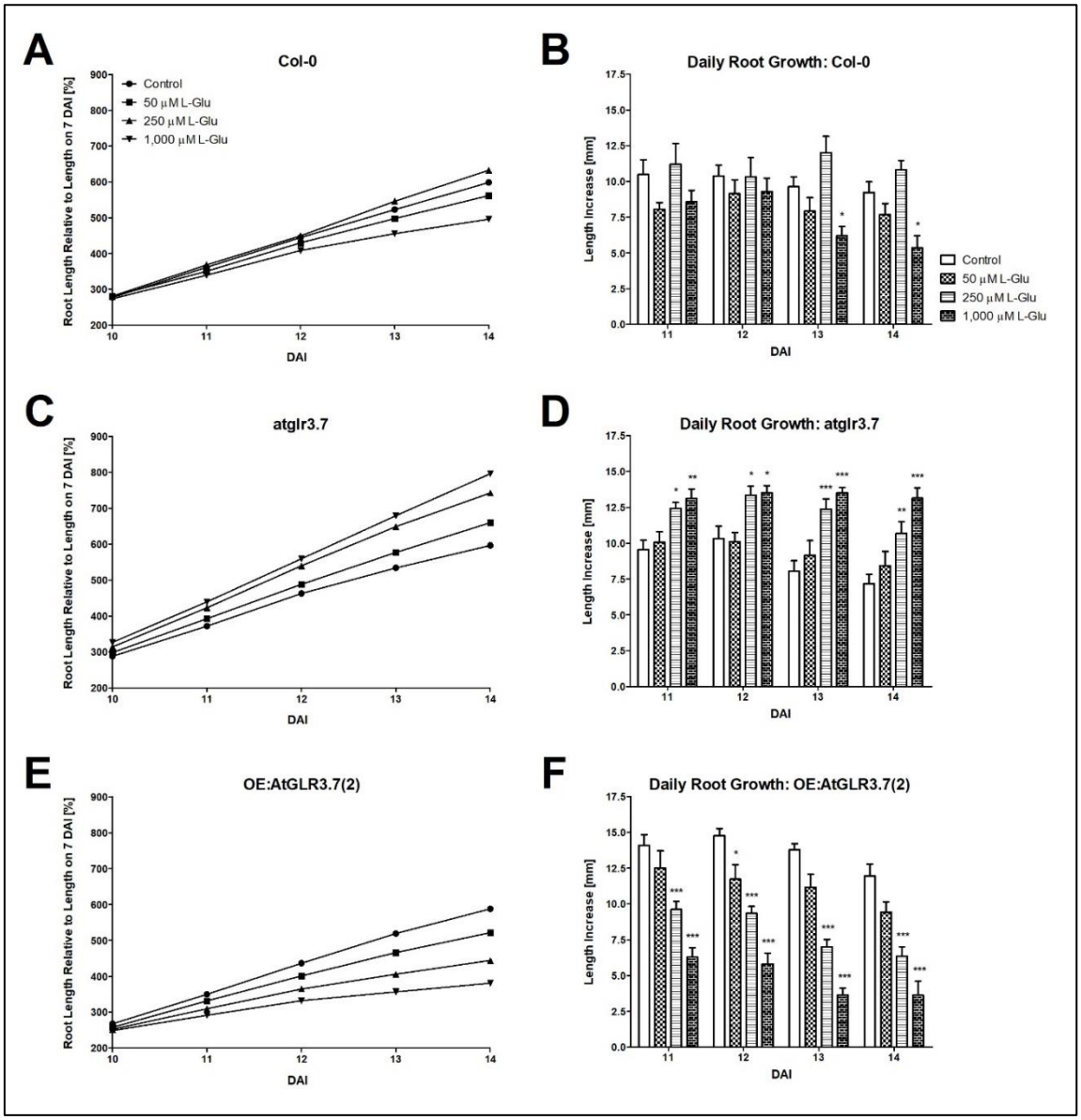

Figure-9. Relative and Daily Root Growth in Response to Rising L-Glutamate Concentrations. Wildtype and transgenic *Arabidopsis thaliana* were grown under standard conditions on ½ MS agar and transferred to L-glutamate-containing agar plates (0, 50, 250 and 1,000 µM) on 7 DAI. Relative root growth refers to the initial root length at the beginning of the exposure to L-glutamate where atglr3.7 responded with a continuing increase in its primary root growth depending on increasing L-glutamate concentrations while OE:AtGLR3.7(2) exhibited the opposite phenotype (A), (C) and (E). Daily root growth is depicted during an advanced exposure to L-glutamate (11-14 DAI) (B), (D) and (F). Statistical analyses among genotypes were conducted using a two-way ANOVA and Bonferroni's *post hoc* test; n = 7-9. Asterisks indicate significant deviations from Col-0 with *$\rho < 0.05$, **$\rho < 0.01$ and ***$\rho < 0.001$. Error bars indicate SE.

4. Discussion

In this work, three separate *Arabidopsis thaliana* overexpression lines were constructed to compare different growth levels of *AtGLR3.7* with wildtype plants as well as with the knockout line atglr3.7. All transgenic plant lines had Col-0 as a genotypic background allowing for an exclusive investigation of the AtGLR3.7-mediated effects on Arabidopsis plant development and physiology. The *AtGLR3.7* overexpression lines were chosen based on their divergent gene expression degrees in order to have an overexpression gradient and to exclude the effect of a potential insertion of the coding sequence into other functional genes within the genome of *Arabidopsis thaliana*. The latter could affect the plant phenotype aside from the desired alterations originating from an *AtGLR3.7* overexpression.

Seed development in *Arabidopsis thaliana* starts after the double fertilization of a central cell (twice haploid) and the egg cell (haploid) in the maternal ovule. The maternal integuments of the ovule develop into the seed coat whereas the fertilized central cell becomes the triploid endosperm, and the fertilized egg cell forms a diploid embryo. The seed development itself takes place in two phases: (I) embryogenesis (syncytial stage) and (II) maturation. The process of endosperm development is primarily made up of several mitotic cycles/nuclear divisions that lead to the formation of a syncytium. A comparison of the different seed sizes between wildtype and the transgenic Arabidopsis plant lines showed a marked increase in seed area for all *AtGLR3.7* overexpression lines while the *AtGLR3.7* knockout line exhibited a reduced seed extension (major, minor axis) and seed perimeter (

Table-1), (Figure 1) During endosperm development, an increase in mitotic cycles of the overexpression lines could have caused an enlargement of the endosperm that would become replaced by the growing embryo in later developmental stages. The reduced seed size in atglr3.7, on the other hand, could be a result of a premature endosperm cellularization leading to a reduced endosperm volume. Luo *et al.*, 2005, could shows that the knockout of the LRR receptor kinase IKU2, which is solely expressed in the endosperm could cause a reduction in seed size. An alternative explanation for the observed *AtGLR3.7*-mediated phenotype could be based on an increase in cell divisions of the integuments. An intensification of cell division activity of these compartments would create a larger space for a development of the endosperm and the embryo. A thorough analysis of the different seed compartments such as the integuments/seed coat, the endosperm and the embryo as well as their development during seed maturation are needed for a better understanding of the developmental processes affected by varying *AtGLR3.7*.

The root system of *Arabidopsis thaliana* is typical for a dicot plant where at the beginning of the root development a pronounced primary root grows out of the embryo. The primary root is afterwards accompanied by several lateral roots that emerge from the root's pericycle. Eventually, adventitious roots are formed during the final stages of the plant development (Hochholdinger and Zimmermann, 2008). Observations of the root growth in *AtGLR3.7* knockout, overexpression and wildtype plant lines revealed strong variations of the primary root length (Figure 2). The overexpression lines show an increase in primary root length. OE:AtGLR3.7(1) was characterized by a root phenotype comparable to Col-0 in all tested parameters, (

Table-2). OE:AtGLR3.7(3) and -(2) exhibited the longest primary roots with significantly increased root diameters while OE:AtGLR3.7(3) exhibited a boost in growth throughout the time course (Figure 3B). In contrast, the knockout line atglr3.7 displayed a shortened primary root and a significant reduction in daily root growth especially on 7 DAI. These findings are in accordance with the observations regarding differences in primary root length which were most pronounced in early plant development stages. The measured deviations were mitigated in later growth stages in which OE:AtGLR3.7(3) showed an almost similar daily growth as Col-0.

The root architecture, apart from the primary root growth/length, however, was alike in all tested plant lines (

Table-2). The frequency of lateral root formation correlated with the effective primary root length. Here, a longer root harboured more secondary roots but the density of additional roots on the primary root was comparable in the different Arabidopsis genotypes. However, the *AtGLR3.7* knockout line was characterized by a minor increase in secondary root density that was likely due to the reduction in primary root growth rate. It seems that the formation of new lateral roots did not parallel the growth process of the primary root in this genotype. Observations of the *AtGLR3.7* overexpression lines showed a growth-stimulating effect for all above-ground tissues apart from the already described enhanced root growth. As a rough estimation, the total plant biomass increased significantly depending on the *AtGLR3.7* expression level in all three overexpression lines while it was reduced in *AtGLR3.7* knockout line.

Detailed measurements revealed an overall growth boost of the rosette-forming leaves in terms of absolute rosette area and radial expansion in OE:AtGLR3.7(2) and to a lesser extent also in OE:AtGLR3.7(3) and -(1) (Figure 4A and table 3). Interestingly, all three overexpression lines displayed an accelerated rosette growth in early developmental stages, but this enhanced growth was only steady in OE:AtGLR3.7(2) whereas OE:AtGLR3.7(3) appeared to suffer from growth impairments at an advanced plant age (Figure 5). These observations were similar to the investigated stem growth where OE:AtGLR3.7(2) displayed a steady growth acceleration while the initial growth boost in the other two overexpression lines abated over time until the final stem length was comparable to Col-0. The slightly enhanced growth characteristics in OE:AtGLR3.7(1) as well as the minor growth retardations in atglr3.7 were in accordance with the established *AtGLR3.7* expression gradient hypothesis. A comparison of the various strains grown in darkness shows a clear difference pointing the role of GLR in plant growth under darkness (Figure 6). Interestingly a significant different was noticed at the level of the root with an insignificant different at the level of the hypocotyl (Figure 7).

Glutamate as a potential major activator of GLRs was externally applied to transgenic and wildtype Arabidopsis plants to measure its effect on primary root growth. Here, significant variations were detected between Col-0, atglr3.7 and OE:AtGLR3.7(2) (Figure 8). Increasing L-glutamate concentrations had the tendency to slow primary root growth. The ability of glutamate to inhibit root growth was already described by Walch-Liu, *et al.* [16] where a

concentration of 1,000 µM L-glutamate diminished primary root length significantly. Although concentrations as high as 1,000 µM were used in the experimental setup for this work, only minor reductions in daily root growth were observed and the more pronounced inhibition of daily root growth begun relatively late on 13 to 14 DAI in wildtype Arabidopsis. The enhanced sensitivity to the inhibiting effect of increasing L-glutamate concentrations was more obvious at higher concentrations of 250 and 1,000 µM where reductions of -25 and -45%, respectively, were detected. Furthermore, an investigation of the daily root growth in response to increasing L-glutamate concentrations revealed that OE:AtGLR3.7(2) responded much earlier with a root growth inhibition than Col-0 (Figure 9E, F). Both observations argue for a higher susceptibility of this transgenic plant line probably due to an excess of *AtGLR3.7* within the plant.

Since L-glutamate caused a suppression of root growth in Col-0 as well as in OE:AtGLR3.7(2) and considering the enhanced susceptibility to this inhibitory effect in the overexpression line, the *AtGLR3.7* knockout line was expected be insensitive to the L-glutamate-mediated reductions in primary root growth. However, increasing amino acid concentrations led to a concentration-dependent increase in primary root growth in atglr3.7 (Figure 9B). Detailed analysis of the daily root growth and a relative root growth comparing the initial root length before the transfer to glutamate-containing agar plates, showed a strong increase in root growth especially at higher concentrations of 250 and 1,000 µM.

Interestingly, a concentration of 250 µM L-glutamate led to a restoration of the primary root growth phenotype in the *AtGLR3.7* both knockout and overexpression line. This finding could indicate a physiologically effective concentration of L-glutamate within the apoplast of plants participating in root growth regulations. It has already been established that naturally occurring Arabidopsis ecotypes show different sensitivities to L-glutamate-mediated inhibitions of their primary root [16]. It is possible that these variations in glutamate susceptibility have their origin in varying expression levels of glutamate receptors such as AtGLR3.7 as it appears in this work where an excess of *AtGLR3.7* conferred a higher sensitivity against L-glutamate than in Col-0.

The mechanism in which elevated L-glutamate concentrations reduce root growth likely involves a diminished RAM size associated with lowered auxin concentrations at the root tip [16]. Since *AtGLR3.7* seems to affect meristematic activities, too, it is conceivable that local auxin maxima could be affected by a misexpression of this glutamate receptor also in the here tested transgenic plant lines.

Another theory implies that atglr3.7 is only a knockdown plant line where still minor quantities of *AtGLR3.7* would be translated. In this case, exogenously applied L-glutamate concentrations in the apoplast would compensate the deprivation of AtGLR3.7 at the PM by increasing the ligand receptor ratio. An application of additional L-glutamate to the root would translate into an imbalance of this system causing root growth reductions in Col-0 and more severely in plants overexpressing *AtGLR3.7* while the same elevated L-glutamate concentrations could compensate the quantitative reduction of AtGLR3.7 in atglr3.7. The normal root growth phenotype could be restored in this plant line by the necessary concentration of exogenously applied L-glutamate. Following this hypothesis, a strictly regulated balance of available and responsive AtGLR3.7 as well as precise concentrations of L-glutamate within the apoplast would govern the meristematic activities within the root tip.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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