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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Involved or not involved - the role of the glutamate receptor 2.5 gene in root development under stress

Promotor :
dr. ir. Tony REMANS

Promotor :
Prof.dr. BRIAN FORDE

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Proefschrift ingediend tot het behalen van de graad van master in de biomedische wetenschappen

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.

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List of Abbreviations

A. thaliana	*Arabidopsis thaliana*
Bp	Base pairs
Cd	Cadmium
cDNA	Complementary deoxyribonucleic acid
$CdSO_4$	Cadmium sulphate
Col	Columbia
Cu	Copper
$CuSO_4$	Copper sulphate
dNTP	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLRs	Glutamate-like receptor homologs
iGluRs	Ionotropic glutamate receptors
KCl	Potassium chlorite
K_2SO_4	Potassium sulphate
LR	Lateral root
L-Glu	L-glutamate
Gln	Glutamine
MAPK or MPK	Mitogen-activated protein kinase
MAPKK or MKK or MEK	MAPK kinase
MAPKK or MEKK	MAPK kinase kinase
MES	2-(N-morpholino)ethanesulfonic acid
NW	North West
PCR	Polymerase chain reaction
PR	Primary root
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative PCR
TAE buffer	Tris-Acetate-EDTA
TAIR	The arabidopsis information resource
UV	Ultraviolet
Zn	Zinc
$ZnSO_4$	Zinc sulfate

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This page will be dedicated to thank all the people, from Hasselt or from Lancaster, who have helped me making this senior practical training memorable.

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Abstract

Anthropogenic activities, like mining and industrial activities, agricultural use of phosphate fertilizers, metal-containing pesticides and fungicides and sewage sludge, have become a worldwide problem as they have led to environmental contamination. The main problem is that metals are not biodegradable and persist in the soil unlike many organic pollutants, which degrade to carbon dioxide and water.

This contamination causes and effect on plant roots, because they are the first parts of the plants that come in close contact with these stresses. Plants lack mobility, for this reason they possess a high plasticity in root development which comes from the sophisticated mechanism for sensing and responding to environmental stimuli. In this way, they can easily adapt to large spatial and temporal changes in the availability of water and mineral ions and stimulate root growth, primary and lateral root growth.

Previous research showed that amino acids can induce a morphogenic response although they are an important nitrogen source for plants. This response is the inhibition of the primary root growth and an increase in lateral root growth. The same observations were made for phenotypic responses to metals. But the question which genes and pathways are involved is still not clear.

In this research a candidate gene for primary root growth inhibition is investigated: this is the *AtGLR2.5* gene, belonging to the family of glutamate receptor-like proteins (GLRs). In *Arabidopsis thaliana*, 20 different *AtGLRs* genes are indentified where 15 of the 20 *AtGLR* genes are expressed throughout the plant and 5 of them are root-specific. The candidate gene has four different splicing variants: *AtGLR2.5a*, *AtGLR2.5b*, *AtGLR2.5c* and *AtGLR2.5d*. Previous unpublished data showed that the transformation of a *glr2.5* mutant with the *AtGLR2.5c* cDNA is sufficient to restore L-glutamate sensitivity. This leads to the first hypothesis that this splicing variant could be involved in the impairment of the primary root growth under L-glutamate stress, and in the second hypothesis, its involvement in metal stress is explored; both investigated in this research project.

The results of this research project indicated a primary root growth under the L-glutamate, copper, cadmium and zinc treatments. The gene expression of the splicing variant *AtGLR2.5c* was also measured under the different treatments. Analysing the data showed that the splicing variant *AtGLR2.5c* was not involved in the primary root growth under L-glutamate stress, but it could be involved in the primary root growth under metal stress.

Samenvatting

Antropogene activiteiten, zoals industriële activiteiten, gebruik van fosfaat meststoffen, metaalbevattende pesticiden en fungiciden in de landbouw, zijn een wereldwijd probleem vermits ze leiden tot de contaminatie van het milieu. Het grootste probleem is dat metalen niet biologische afbreekbaar zijn en persistent in de bodem blijven zitten in tegenstelling tot organische polluenten, welke afbreken tot koolstofdioxide en water.

Deze contaminatie veroorzaakt een effect op de plantenwortels vermits deze de eerste delen zijn die in contact komen met deze stress factoren in de bodem. Planten hebben geen mobiliteit en hierdoor hebben ze een verhoogde plasticiteit van het wortelstelsel ontwikkeld welke ontstaan is door een gesofisticeerd mechanisme voor het voelen en reageren op omgevingsstimulansen. Op deze manier kunnen ze zich gemakkelijk aanpassen aan grote spatiale en temporele verandering in de aanwezigheid van water en minerale ionen en hierdoor wortelgroei; primair en lateraal, stimuleren.

Eerder onderzoek heeft aangetoond dat aminozuren een morfogenetische responsie kunnen induceren ook al zijn ze een belangrijke bron van stikstof voor planten. Deze responsie is de inhibitie van de primaire wortel groei en een verhoging in laterale wortel densiteit. Dezelfde observaties zijn gemaakt voor de fenotypische responsie geïnduceerd door metalen. Maar de vraag blijft nog steeds welke gene en pathways betrokken zijn in dit mechanisme.

In dit onderzoek werd een kandidaat gen voor primaire wortelgroei inhibitie onderzocht: het *AtGLR2.5* gen,behorend tot de familie van de glutamaat receptor gelijkende proteïnen (GLRs). In *Arabidopsis thaliana*, zijn 20 verschillende *AtGLRs* genen geïdentificeerd waarvan 15 tot expressie komen doorheen de plant en 5 specifiek in de wortels. Het kandidaat gen komt voor in 4 isovormen: *AtGLR2.5a*, *AtGLR2.5b*, *AtGLR2.5c* and *AtGLR2.5d*. Eerder ongepubliceerde data toonde aan dat de transformatie van een *glr2.5* mutant met *AtGLR2.5c* cDNA voldoende was om glutamaat sensitiviteit te herstellen. Dit leidde tot de eerste hypothese dat deze isovorm kan betrokken zijn in de gereduceerde primaire wortelgroei onder L-glutamaat stress en een tweede hypothese, waar dit betrokken is in metaal; beide zijn onderzocht in dit onderzoeksproject.

De resultaten van dit onderzoeksproject toonde een inhiberend effect van de primaire wortelgroei aan onder de verschillende groeicondities. De gen expressie van de isovorm *AtGLR2.5c* was ook gemeten onder de verschillende blootstellingen. De data analyse toode aan dat deze isovorm niet betrokken is in de primaire wortelgroei onder L-glutamaat blootstelling maar de isovorm kan betrokken zijn in de primaire wortelgroei onder metaalstress.

1 Introduction

During the past century, some anthropogenic activities, like mining and industrial activities, agricultural use of phosphate fertilizers, metal-containing pesticides and fungicides and sewage sludge, have become a worldwide problem as they have led to environmental contamination; the increase in metal concentrations in soils (2). The main problem is that metals are not biodegradable and persist in the soil unlike many organic pollutants, which degrade to carbon dioxide and water. Some of those metals such as copper (Cu) and zinc (Zn) are essential micronutrients because they are involved in the physiological processes which leads to normal growth and development. But this is also the reason of their toxicity because in high concentrations they can disturb normal development by adversely affecting biochemical reactions and physiological processes in plants. Other metals such as cadmium (Cd) are nonessential, but can still be taken up by the transport systems and cause an adverse effect on normal plant growth and development. So many soils contain both group of metals; essential and non-essential which can become cytotoxic for plants and disturb normal plant growth when they reach high bioavailable concentrations in the soil (2-4).

One of the other consequences of the modern lifestyle of humans and its growing population is the fact that we need more areas for the development of crops. Crops for the food production but also crops for renewable energy. The heavy polluted areas could be a solution for the growth of different crops. Not for agricultural activities, because the toxic substances in the soil will accumulate in the crops and can bring these potentially toxic metals into our food chain, but for the production of biomass for renewable energy. This would make the soils, that were used for biomass production, available for the growth of crops destined for food production (5).

Those polluted soils will give problems in the development of the crops and in order to improve biomass production, more insight needs to be gained in plant roots or the so called "second green revolution", were roots are considered as mediators of increased nutrient uptake efficiency and drought tolerance. Roots are the first part of the plant that come in direct contact with the nutrients, minerals and other molecules in the soil and for this reason, nutrients have a major influence on the root phenotype. Another important aspect of plants is that they adapt to their environment because they lack mobility. Therefore they possess a high plasticity in root development which comes from the sophisticated mechanism for sensing and responding to environmental stimuli, so that they can easily adapt to large spatial and temporal changes in the availability of water and mineral ions and can stimulate root growth, primary and lateral root growth. Of course, some plants are less affected than others by the substances in the soil because they have developed some resistance; like mutations in genes involved in root growth. (5-8).

Like previously stated, in those contaminated soils, there are a number of biotic and abiotic stresses from which plants cannot escape because of their immobility, for example amino acids and metals are stresses which can induce a morphogenic response. Amino acids are present in the soil and can cause stress although they are an important nitrogen source for plants. One of those amino acids is glutamate, which has a role as neurotransmitter in the central nervous system in animals. Glutamate can be present in high concentrations in decomposing organic matter: animal and plant tissue contain free glutamate. Roots growing in those patches will respond to the presence of glutamate with a

decrease in primary roots and increase in lateral root density. Previous research showed that only the L-stereoisomer has an influence on the primary and lateral roots (8, 9). On the other hand, like mentioned in the first paragraph, some metals, like cadmium, copper and zinc can become phytotoxic in high concentrations even though zinc and copper are essential in low concentrations and this metal toxicity can disrupt different processes in plants and induce for instance: chlorosis or necrosis and eventually lead to reduced growth, mostly seen in the root phenotype which has a reduction in primary root elongation and an increase in lateral root density (5, 10). The question remains which genes and signalling pathways could be involved and eventually lead to those morphological changes in plants.

One group of genes that could be potential candidates in the behavioural responses in plant roots are the *AtGLR* genes belonging to the family of glutamate receptor-like proteins (11). In *Arabidopsis thaliana*, 20 different *AtGLRs* genes are indentified and divided into three distinct phylogenetic clades based on parsimony analysis with bacterial amino acid binding proteins as out-groups (12). Also expression studies have shown that 15 of the 20 *AtGLR* genes are expressed throughout the plant and 5 of them are root-specific (8, 13). These genes code in plants for glutamate-like receptor homologs (GLRs) of mammalian ionotropic glutamate receptors (iGluRs) which are involved in neurotransmission. Previous research showed that the GLRs are involved in various biological processes, such as C/N balance (14), photosynthesis (15), responses to abiotic stress (16), plant-pathogen interaction (17), regulation of cellular Ca^{2+} kinetics (17), pollen tube growth (18) and root morphogenesis (19), which can imply that GLRs are indeed capable of perceiving and transducing amino acid signals like the iGluRs in animals.

The structure of plant iGluRs (Fig. 1) is composed of a ligand binding domain (LBD), which consists of two subdomains, GlnH1 and GlnH2, an additional ATD and a channel forming domain, which consists of three complete trans-membrane domains (M1, M3 and M4) and one partial trans-membrane domain (M2) that forms a pore-loop (P-loop) structure. M1, M3 and the P-loop form a structure that resembles the tetrameric potassium channels such as KcsA, with inverted topology. These plant GLRs are also amino acid-activated channels, because there is now electrophysiological evidence obtained *in planta* that at least in some instances, GLRs can act as Glu-gated Ca^{2+} channels (17, 20). This proves that they would be able to sense amino acids at the exterior of the membrane in which the GLRs are localized and could lead to the theory that the GLR family is involved in cell-to-cell communication in plants for root development by sensing L-glutamate and maybe metals too in the soil (1).

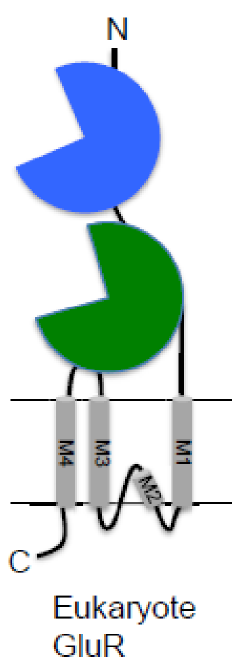


Figure 1: Schematic representation of an eukaryote glutamate receptor. The proposed origins of ligand binding domain (LBD, green) and amino terminal domain (ATD, blue), and the channel domain (1).

In mammals, MAP kinase pathways play an important role in the signalling of the iGluR-mediated response to L-glutamate. And these MAP kinase pathways are also involved in metal stress for different plant species. These pathways are composed of three different protein kinase modules: MAPKK kinases (MAPKKK), MAPK kinases (MAPKK or MKK) and MAP kinases (MAPK or MPK). First MAPKKKs, serine/threonine kinases, are activated and they phosphorylate MAPKKs via serine/threonine residues in the S/T-X_5-S/T motif. Then the MAPKKs activate MAPKs, also through phosphorylation, but in both tyrosine and serine/threonine residues in the TXY motif. And those MAPKs are the kinases that phosphorylate different substrates like transcription factors, splicing factors and other protein kinases involved in hormone responses, plant innate immunity and biotic and abiotic stress. In *A. thaliana*, 60 MAPKKKs, 10 MAPKKs and 20 MAPKs have been identified and their main function is translating external stimuli into cellular responses (2, 21, 22).

This project aims to obtain more fundamental knowledge on the response of plants to different stresses: L-glutamate, cadmium, copper and zinc and which eventually leads to the inhibition of the primary root growth and increase in lateral root growth. *A. thaliana* has 20 different *AtGLR* genes and one of them; the *AtGLR2.5* gene, could be the gene that is involved in the inhibition. Preliminary data from a systematic analysis of T-DNA insertion mutants showed that a mutant of this gene had a reduced sensitivity to high concentrations of L-glutamate. It is expressed throughout the plant but highly in the root cap, which would be consistent with a possible role in initiating the root's response to external L-glutamate (23). Previous unpublished data showed that this gene may be a negative regulator of L-glutamate-induced Ca^{2+} fluxes at the root tip and has four different splicing variants; *AtGLR2.5a* (2605 bp), *AtGLR2.5b* (1451 bp), *AtGLR2.5c* (1250 bp) and *AtGLR2.5d* (512 bp), all of which encode incomplete GLR proteins (Fig. 2). The different splicing variants occur by alternative splicing (AS); a regulated process during gene expression that results in a single gene coding for multiple proteins (24).

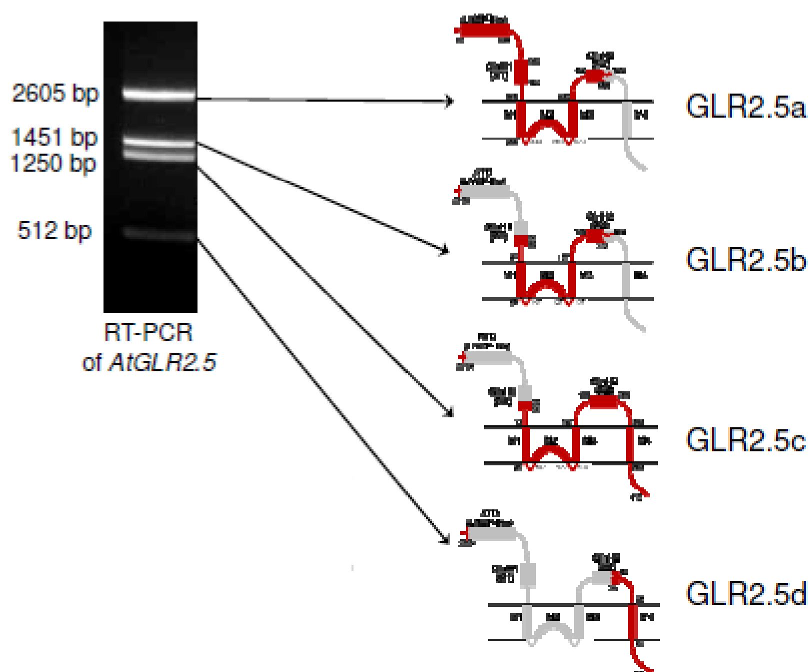


Figure 2: Overview of the four different splicing variants of the *AtGLR2.5* gene.

More specifically, this research project will focus on one particular splicing variant of the *AtGLR2.5* gene; the *AtGLR2.5c* splicing variant, because previous unpublished data showed that the transformation of a *glr2.5* mutant with the *AtGLR2.5c* cDNA is sufficient to restore L-glutamate sensitivity. A first hypothesis states that the expression of the *AtGLR2.5c* splicing variant could be related to the L-glutamate sensitivity and induces the observed root phenotype: inhibition of the primary root growth and increase in lateral root density. This similar root phenotype is also observed under metal stress, which leads to our second hypothesis that the expression of the *AtGLR2.5c* splicing variant could be related to copper, cadmium and zinc sensitivity. All this research will be performed in a vertical agar plate system which easily shows the root system of *A. thaliana* and different root growth parameters are already quantified. *A. thaliana* is a model organism for environmental studies because of the vast knowledge on intrinsic root developmental programmes and the small root system is easily to quantify (6).

2 Materials & Methods

2.1 Culture conditions

Plant material. The *Arabidopsis thaliana* L. wild-type ecotypes used in the experiments were the Columbia (Col-0 and Col-8), the C24 and the North West (NW), all available at the university. The different mutants used, all in a Col-8 background, were the *MKK1*- (SALK_027645), the *MKK2*- (CS 66015), the *MKK3*- (SALK_051970), the *MKK2/3*- (obtained from other researchers), the *MEKK1/2/3*- (available at Lancaster University), the *MKK7*- (CS 104671), the *MKK9*- (SALK_017378) and the *MKK10* (CS 825626) mutant.

Plant growth. Seeds were placed in a cone-shape folded filter paper (Whatmann hardened ashless type 542) that was closed with a plastic paper clip, then surface-sterilised in 0.1% sodium hypochlorite solution (w/v) and 0.1% Tween 80 for 1 minute and washed four times for 5 minutes in sterile distilled water, followed by drying in a laminar air flow. Afterwards the seeds were sown at the top of 12-x 12-cm vertical nursery agar plates containing 50X diluted Gamborg's B5 macro- and micronutrients: 0.1 mM KCl, 0.04 mM $MgSO_4.7H_2O$, 0.02 mM $CaCl2.6H_2O$, 0.022 mM NaH_2PO_4 and 0.9 µM $MnSO_4.4H_2O$, 90 nM KI, 0.97 nM H_3BO_3, 0.14 nM $ZnSO_4.H_2O$, 2 nM $CuSO_4$, 20.6 nM $Na_2MoO_4.2H_2O$ and 2.1 nM $CoCl_2.6H_2O$, 3.6 mM Fe-EDTA, 0.5 g MES (2-[N-Morpholino]ethanesulfonic acid), 5 g sucrose, 10 g plant tissue culture agar (Lab-M, Bury, UK) for one litre. The pH was adjusted to 5.7 with KOH before addition of sucrose and agar. The nursery plates also contained 0.5 mM glutamine (Gln) as nitrogen source that was added after autoclaving the medium. Afterwards plates were closed with parafilm, with three gaps to allow air exchange, and kept in the dark for two days at 4°C (imbibition). Then nursery plates were placed vertically in a culture room for five days under a 12h/25°C day- and 12h/21°C night regime and an average light intensity of 140 µmol m^{-2} s^{-1} delivered by fluorescent white lamps (Philips). Vertical agar treatment plates contained the same medium composition as above and additionally appropriate amounts of filter-sterilized (0.2 µm) L-Glu, KCl, $CdSO_4$, $CuSO_4$, $ZnSO_4$ and K_2SO_4 solutions were mixed into the medium. One cm of agar was removed at the top of each treatment plate to create an air gap for the shoots. After five days, a selection of plants with similar primary root length were transferred to the 12-x 12 cm treatment plates. Plates were sealed with parafilm with three air gaps to allow air exchange, and placed vertically in the culture room for 24h or seven days, depending on the experiment, under a 12h/25°C day- and 12h/21°C night regime and an average light intensity of 140 µmol m^{-2} s^{-1} delivered by fluorescent white lamps (Philips).

2.2 Specific conditions and plant material in the different experiments, and modifications from the above described general protocol

Glutamate sensitivity of ecotypes. The five-day-old seedlings in this experiment were transferred for 24h or seven days to treatment plates containing various concentrations of glutamate. The different ecotypes; NW, C24 and Col-8 were exposed to 0 mM and 50 µM L-Glu. The Col-8 ecotype was also exposed to 1 mM L-Glu because previous experiments showed that this ecotype is less sensitive for glutamate under lower concentrations.

L-glutamate and metal sensitivity in Col-0. The five-day-old seedlings in this experiment where transferred for 24h or seven days to treatment plates containing medium mixed with 1 mM L-Glu, 5 µM

$CdSO_4$, 10 μM $CuSO_4$ or 75 μM $ZnSO_4$. K_2SO_4 was added to obtain similar concentrations of SO_4^{2-} in the medium that were supplemented with metal-sulphates.

Glutamate sensitivity of MAPK signalling mutants. In this experiment, the seeds were first surface-sterilized in absolute ethanol for 1 minute and followed by 10 minutes in 20% Sodium Hypochlorite solution 0.01% Triton X100 and at least seven washes in sterile distilled water for 20 minutes. Followed by drying in a laminar air flow. Afterwards the seeds were sown on round 90 mm nursery Petri dishes containing 50X diluted Gamborg's B5 macro- and micronutrients, as above. After incubation period for two days at 4°C, the plates were placed vertically in a culture room at ~21°C with a 16h/8h light/dark regime and a light intensity of ~100 μmol m^{-2} s^{-1} delivered by fluorescent white lamps (Crompton). Five-day-old seedlings were transferred to treatment plates which also contained 50X diluted Gamborg's B5 macro- and micronutrients but agar was replaced by 10g phytagel and 2 ml 0.5 M $CaCl_2$ and 5 ml 0.5 M $MgCl_2$ was added to solidify the phytagel (Sigma-Aldrich, UK). Two mM L-Glu and 2 mM KCl were added to the treatment media and control plates respectively to get the same K concentration. The plates were placed vertically in a culture room at ~21°C with a 16h/8h light/dark regime and a light intensity of ~100 μmol m^{-2} s^{-1} delivered by fluorescent white lamps (Crompton).

2.3 RNA extraction and gene expression analysis

RNA extraction. The roots of the seedlings, grown for 24h on treatment plates, were harvested and immediately frozen in liquid nitrogen. Then the root samples were homogenized in 2 ml tubes, which contained one tungsten bead (Ø 2.5 mm), for 2 minutes at 30 Hz with the Retsch Mixer Mill MM200 (Düsseldorf, Germany). Total RNA was extracted from the homogenised tissues with the Nucleospin RNA XS kit (Macherey-Nagel, Germany) according to the manufacturer's protocol except for step 8, where 3 μl reconstituted rDNase and 27 μl reaction buffer for rDNase was replaced by 6 μl reconstituted rDNase and 24 μl reaction buffer for rDNase and the incubation period was increased from 15 minutes to 30 minutes.

cDNA synthesis. The concentration of RNA samples were determined with the Nanodrop 1000 (Thermo scientific, Wilmington, DE, USA), adjusted to the same concentration with RNase-free water and measured again to homogenize RNA input for the cDNA synthesis reaction. Out the total RNA, 500 ng was used to make cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo scientific, USA) according to the manufacturer's protocol and afterwards stored at -20°C.

Full length cDNA PCR of GLR2.5 and analysis. The *AtGLR2.5* gene was PCR amplified by two different gene-specific primer pairs which were designed based on the sequence of *AtGLR2.5* obtained from the TAIR database (Arabidopsis.org)(Appendix 1). The first pair of gene-specific primers were designed from the first putative startcodon in the *AtGLR2.5* gene with forward primer 1 (5'-atgtctcttttccatcatctc-3') and reverse primer (5'-ctagagtttaggtttgactat-3'). The second pair of gene-specific primers were designed from the second putative startcodon in the *AtGLR2.5* gene with forward primer 2 (5'-atggcttcaagacaaggattg-3') and the same reverse primer as above. Both pairs were used to amplify the full length coding sequence of *AtGLR2.5*. The cDNA was amplified using the Phusion High-Fidelity DNA polymerase kit (Thermo scientific, USA) and each PCR reaction contained 36 μl RNase-free water, 10 μl 5X Phusion HF Buffer, 1 μl dNTP mix (10 mM each), 0.25 μl forward primer (final concentration: 0.5 μM), 0.25 μl reverse primer (final concentration: 0.5 μM), 5 μl cDNA and 0.5 μl

Phusion DNA polymerase (0.02 U/µl). PCR was performed with the following temperature profile: an initial cycle at 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds, 53°C for 20 seconds, 72°C for 100 seconds and a final cycle at 72°C for 10 minutes. Afterwards the PCR amplicons were visualized on a 0.8% agarose gel (50 ml 1 X TAE buffer, 0.4 g Ultrapure Agarose and 5 µl Gelred) that was run for approximately two hours at 90V. The bands were visualised under a UV-light and the ladder used was a 1 Kb Plus ladder (Invitrogen).

Quantitative gene expression analysis. Real-time PCR was performed in an optical 96-well plate and the Applied Biosystems 7500FAST instrument (Life technologies, Foster city, CA, USA). Universal cycling conditions were used, followed by the generation of a dissociation curve to check for specificity of amplification. Each 10 µl PCR reaction contained 5 µl of 2xSYBR green Master Mix (Life technologies, USA), 300 nM forward primer, 300 nM reverse primer, 2 µl template cDNA and 2.4 µl RNase-free water. The primer pairs used for quantitative gene expression were; the primer pair to obtain all the isoforms of the *AtGLR2.5* gene (forward primer (5'-tgcttcacccaatctaccaa-3') and reverse primer (5'-caaagctgtgtcgacttcca-3')), the primer pair for the *AtGLR2.5a* splicing variant of the *AtGLR2.5* gene (forward primer (5'-tcgggtcaagactcttttcg-3') and reverse primer (5'-caagcttgattcccccatta-3')), the primer pair for the *AtGLR2.5b* splicing variant of the *AtGLR2.5* gene (forward primer (5'-ttaatggtggcagcagaaga-3') and reverse primer (5'-ttcacaagtttggtgcgtaca-3')), the primer pair for the *AtGLR2.5c* splicing variant of the *AtGLR2.5* gene (forward primer (5'-cgttcgatgaggtcgcttat-3') and reverse primer (5'-gtggaaatgcaaagccaaag-3')),and the primer pair for the *AtGLR2.5d* splicing variant of the *AtGLR2.5* gene (forward primer (5'-atggcttcaagacaaggattg-3') and the reverse primer (5'-tctgacaccaatggagatcct-3')) (Appendix 1).
The expression level of the *AtGLR2.5* gene and splicing variant *AtGLR2.5c* were normalised to the geometric average of the expression levels of three reference genes for the glutamate sensitivity of ecotypes study: the SAND family (AT2g28390)(FP, 5'-aactctatgcagcatttgatccact-3'; RP, 5'-tgattgcatatctttatcgccatc-3'), the mitosis protein YLS8 (AT5g08290)(FP, 5'-ttactgtttcggttgttctccattt-3'; RP, 5'-cactgaatcatgttcgaagcaagt-3') and the F-box protein (AT5g15710)(FP, 5'-tttcggctgagaggttcgagt-3'; RP, 5'-gattccaagacgtaaagcagatcaa-3').
Five reference genes were used for the second part of the glutamate sensitivity of ecotypes study: the SAND family (AT2g28390), the mitosis protein YLS8 (AT5g08290), the F-box protein (AT5g15710) and Ubiquitin (AT4g05320)(FP, 5'-ggccttgtataatccctgatgaataag-3'; RP, 5'-aaagagataacaggaacggaaacatagt-3') and EF1a (AT5g60390)(FP, 5'-tgagcacgctcttcttgctttca-3'; RP, 5'-ggtggtggcatccatcttgttaca-3').
And five reference genes were used for the L-glutamate and metal sensitivity in Col-0 study: the SAND family (AT2g28390), the mitosis protein YLS8 (AT5g08290), the F-box protein (AT5g15710), ubiquitin conjugating enzyme (AT5g25760)(FP, 5'-ctgcgactcagggaatcttctaa-3'; RP, 5'-ttgtgccattgaattgaaccc-3') and the TIP41-like (AT4g34270)(FP, 5'-gtgaaaactgttggagagaagcaa-3'; RP, 5'-tcaactggatacccttcgca-3')

2.4 Root analysis

The root systems of the seedlings, that had been growing for seven days on the vertical agar treatment plates and marked daily, were scanned by placing the plates on a conventional flatbed scanner. Digital images were analysed using the Optimas image analysis software (MediaCybernetics, Silver spring, MD). Different root growth parameters were determined for each seedling; the precise coordinates of

the primary root apex after the transfer to treatment plates, length of the primary root (PR), length and number of lateral roots (LR). Primary root growth data were processed in MS Excel.

2.5 Statistics

Statistical analysis was performed with the computer programme R. The normal distribution and homoscedasticity were investigated with the Shapiro-Wilk normality test and the Bartlett test of homogeneity of variances respectively to determine whether a parametric or non-parametric test should be used. For all root growth analysis a parametrical 'two-way ANOVA' was used because there were two variables: genotype and treatment except for the L-glutamate and metal sensitivity in Col-0 study, where a parametrical 'one-way ANOVA' was used because treatment is the only parameter. In order to find significant differences between the mean values a Tukey's HSD(Honest Significant difference) test was used. The gene expression analysis was performed with a parametrical 'two-way ANOVA' for both glutamate sensitivity of ecotypes studies because there were also two variables: genotype and treatment. For the L-glutamate and metal sensitivity in Col-0 study a 'one-way ANOVA' was used, the only parameter is treatment. In order to find significant differences between the mean values a Tukey's HSD(Honest Significant difference) test was also used.

3 Results & Discussion

3.1 Glutamate sensitivity of ecotypes

This research investigated if different ecotypes of *A. thaliana*: NW, C24 and Col-8 had a distinct root phenotype and differed in primary root length as a reaction to L-glutamate exposure and whether there was an influence of L-glutamate on gene-expression of the *AtGLR2.5* gene and its splicing variants in these different ecotypes.

Phenotypic screening and primary root length. For this part of the experiment, five-day-old seedlings of 3 different ecotypes; NW, C24 and Col-8 were transferred to treatment plates for one week which contained 0, 50 µM and 1 mM L-Glu.

The different ecotypes of *A. thaliana*; NW, C24 and Col-8 had distinct root phenotypes in the vertical agar system. C24 had the shorter root phenotype and more lateral roots compared to NW and Col-8, who shared a more similar root phenotype under normal growth conditions. When five-day-old seedlings of each ecotype were transferred to the treatment medium, which contained L-glutamate, the primary roots of all three ecotypes were inhibited after a few days and lateral roots increased in number and length (Fig. 3). The reason for this is the fact that lateral roots are not immediately inhibited by the presence of glutamate. They gain L-glutamate sensitivity later in their development than primary roots (9).

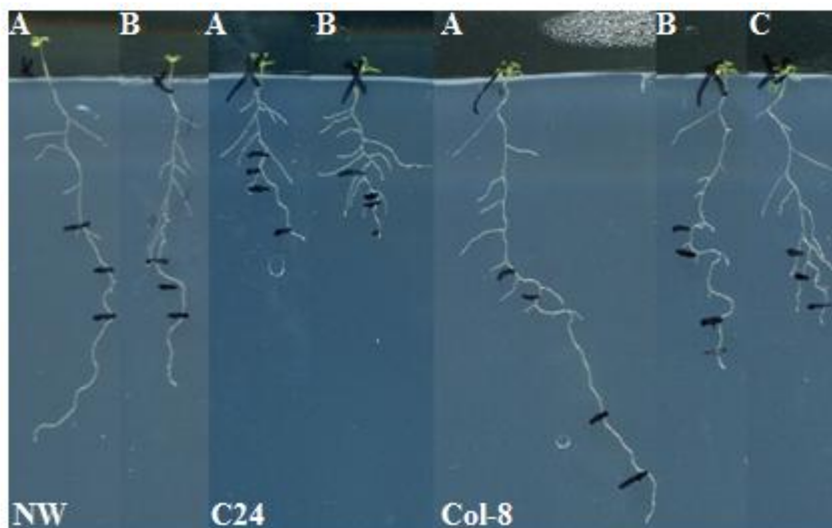


Figure 3: Effect of L-Glu on root phenotype of different eleven-day-old ecotypes of *A. thaliana*. The 3 different wild-type ecotypes; NW, C24 and Col-8 were transferred to treatment plates after five days which contained solid 50 X diluted Gamborg's B5 macro- and micronutrients medium; only medium (A), 50 µM (B) and 1 mM (C) L-Glu. On day zero, one, five and six a mark was set on the vertical agar plate.

Quantitative data obtained from measuring the primary root growth from seedlings after transfer to treatment medium (Fig. 4), confirmed the visual observations made out the root phenotypes shown in figure 3.

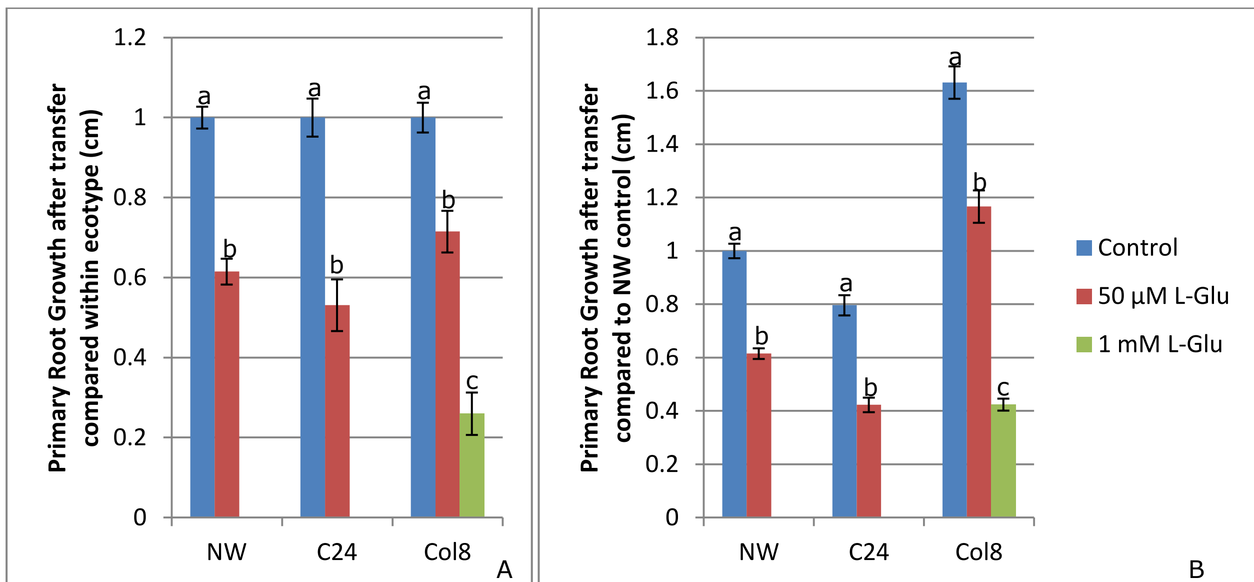


Figure 4: Measurement of primary root growth inhibition in different ecotypes of *A. thaliana* exposed to L-Glu. The primary root growth after transfer to treatment media for one week (in cm) was shown in function of the different L-glutamate concentrations from different eleven-day-old ecotypes of *A. thaliana* exposed to 0, 50 μM and 1 .mM L-Glu, compared within ecotype (A) and compared to NW control (B). Different letters indicate statistically significant differences within ecotype (a, b, c)($P < 0.05$; two-way ANOVA and Tukey's HSD test). For NW; n=9, for C24; n = 14 and Col8; n=12.

Statistical analysis using a 'two-way ANOVA', showed that the treatment condition with L-glutamate had a significant reduction in primary root growth compared to the control condition within each ecotype. The 1 mM L-Glu exposure had a stronger effect on the primary root growth than the 50 μM L-Glu exposure in Col8. When compared to the control conditions of NW, the results were unchanged and primary root growth was inhibited for each ecotype under L-glutamate stress.

Expression of the different splicing variants of *AtGLR2.5*. To check which splicing variants of the whole *AtGLR2.5* gene were expressed in the different ecotypes and to investigate whether there was a difference in splicing variants between ecotypes under L-glutamate exposure, a RT-PCR analysis was performed with two different primer pairs designed from the first- (Fig. 5-1) and the second (Fig. 5-2) putative startcodon. Both primers pairs were designed to amplify the whole *AtGLR2.5* gene and to obtain the four different splicing variants.

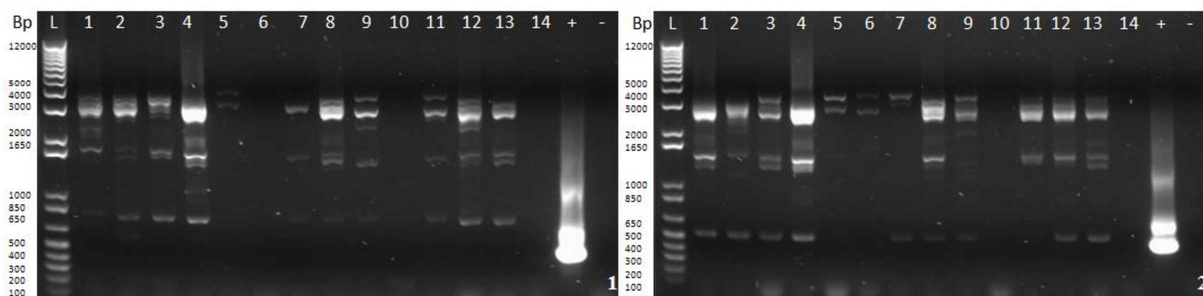


Figure 5: RT-PCR analysis of splicing variants of the *AtGLR2.5* gene under L-Glu exposure in different ecotypes of *A. thaliana*. *AtGLR2.5* gene cDNA was amplified, using two specific primer pairs designed from the first- (Fig. 3-1) and the second (Fig. 3-2) putative startcodon. Duplicate samples: NW grown on control (lane 1, 2) or 50 μM L-Glu (lane 3, 4), C24 grown on control (lane 5, 6) and 50 μM L-Glu (lane 7, 8) and Col-8 grown on control (lane 9, 10), 50 μM L-Glu (lane 11, 12) and 1 mM L-Glu medium (lane 13, 14). Also a positive control for successful RT (+), contained human GADPH control RNA template and GAPDH-specific PCR primers, and a negative control(-), where cDNA template was replaced by RNase-free water, were analysed.

During gel electrophoresis, the different splicing variants were separated according to their fragment size. A 1 Kb plus ladder (L) was used as a reference to identify the different splicing variants from the *AtGLR2.5* gene; *AtGLR2.5a* (2605 bp), *AtGLR2.5b* (1451 bp), *AtGLR2.5c* (1250 bp) and *AtGLR2.5d* (512 bp)(Fig. 2). The different bands obtained in figure 5-2 were of similar size compared to the size of the previously identified splicing variants. The different bands obtained in figure 5-1 were not the size of the previously identified splicing variants. The differences in base pair length came from the fact that for splicing variants in figure 5-2, the same primer pair was used that started from the second putative startcodon and for the splicing variants in figure 5-1,another primer pair was used designed from the first putative startcodon which made the splicing variants 205 bp longer in size (Appendix 1). The data obtained from this RT-PCR differed between duplicates which can be an indication of different RNA input or efficiency of cDNA synthesis, yielding variable cDNA input in the amplification reaction. There was a possibility that the splicing variants differed between the ecotypes and some splicing variants were expressed more than other splicing variants. This could be an indication that L-glutamate had an influence on the expression and/or alternative splicing of the *AtGLR2.5* gene. But to get a more confident quantification of the different splicing variants, a qRT-PCR should be performed with normalisation.

Quantitative gene expression analysis. The quantification of the different splicing variants was performed by a qRT-PCR. The gene expression of all the isoforms (primers make no distinction between isoforms) and the splicing variant *AtGLR2.5c* of the *AtGLR2.5* gene were measured. This to investigate if there was a difference between ecotypes in gene expression of the splicing variants of the *AtGLR2.5* gene in general, the splicing variant *AtGLR2.5c* specifically, and if L-glutamate exposure had an differential influence on this gene expression.

The expression of all the isoforms of the *AtGLR2.5* gene was clearly up-regulated under 50 µM L-Glu exposure for each of the different ecotypes. The 1 mM L-Glu concentration for Col-8 showed a down-regulation of the expression of all the isoforms of the *AtGLR2.5* gene compared to the control conditions (Fig. 6-A). The expression of all the isoforms of the *AtGLR2.5* gene differed between each ecotype, where C24 had the lowest expression and NW and Col-8 had a similar expression. But the expression of all the isoforms of the *AtGLR2.5* gene was still up-regulated for the 50 µM L-Glu treatment for each ecotype (Fig. 6-B). Statistical analysis showed only for the ecotype C24 under L-glutamate exposure a significant difference in gene expression for all the isoforms of the *AtGLR2.5* gene.

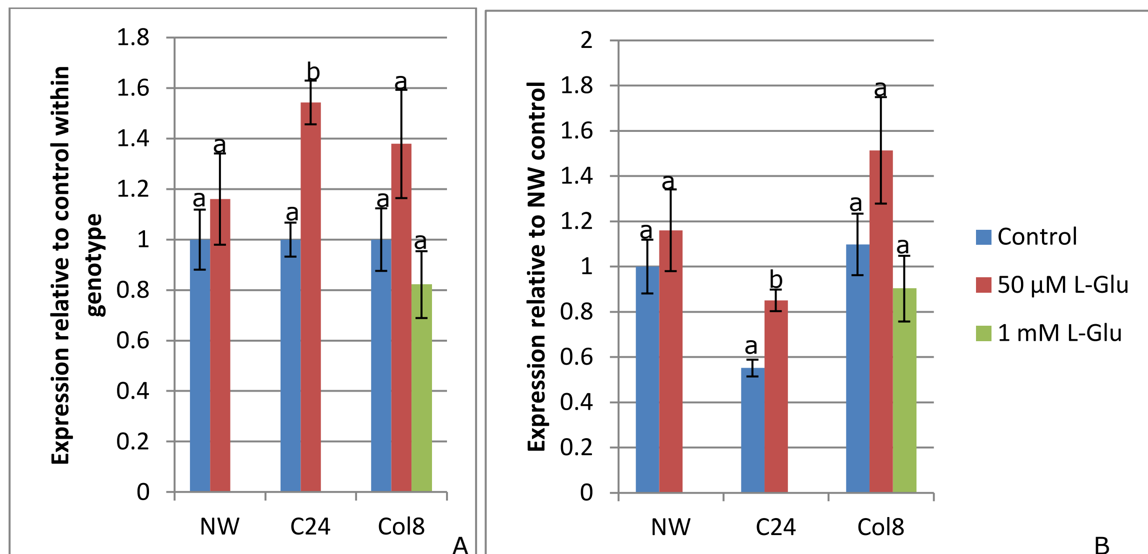


Figure 6:RT-qPCR based gene expression analysis of all the isoforms of *AtGLR2.5* gene in *A. thaliana* under L-Glu treatment. This figure shows the gene expression of all the isoforms of *AtGLR2.5* gene relative to the control within each ecotype (A) and relative to the control of the NW wild-type ecotype (B) when exposed to L-Glu. Different letters indicate statistically significant differences within ecotype (a, b)($P < 0.05$; two-way ANOVA and Tukey's HSD test). For each ecotype, n = 2.

The expression of the splicing variant *AtGLR2.5c* was also investigated and each ecotype exposed to L-glutamate had an increased gene expression for the splicing variant *AtGLR2.5c*. and the 1 mM L-Glu treatment for Col-8 had a higher expression then the 50 µM L-Glu treatment (Fig. 7-A). The gene expression of the splicing variant *AtGLR2.5c* differed between the ecotypes, where C24 had the lowest expression, followed by Col-8 and NW has the highest expression for this splicing variant. Only the gene expression was significantly different for C24 under L-glutamate treatment (Fig. 7-B).

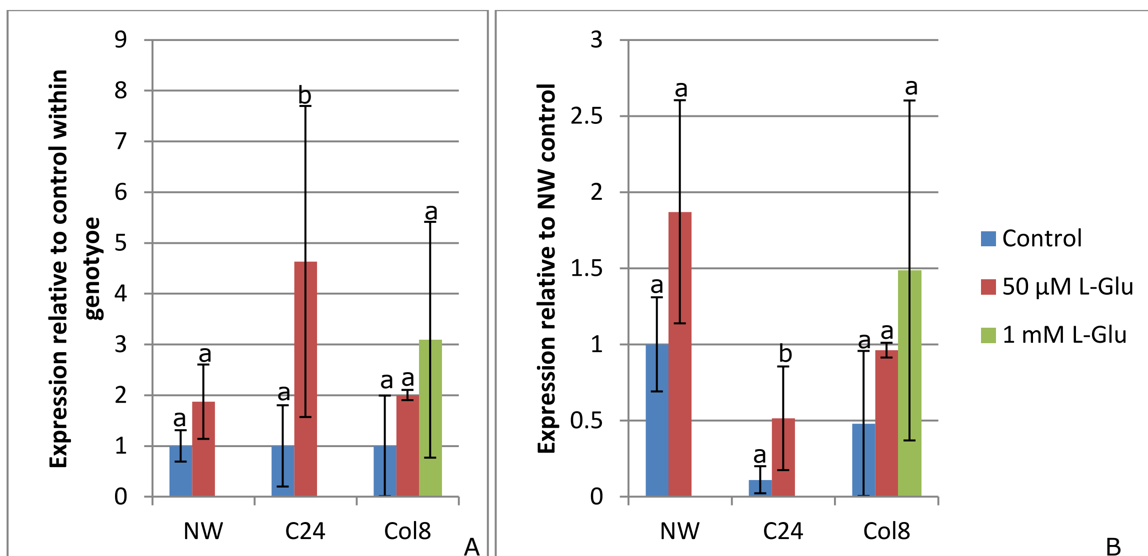


Figure 7: RT-qPCR based gene expression analysis of the splicing variant *AtGLR2.5c* in *A. thaliana* under L-Glu treatment. This figure shows the gene expression of the splicing variant *AtGLR2.5c* relative to the control within each ecotype (A) and relative to the control of the NW wild-type ecotype (B) when exposed to L-Glu. Different letters indicate statistically significant differences within ecotype (a, b)($P < 0.05$; two-way ANOVA and Tukey's HSD test). For each ecotype, n = 2.

The phenotypic screening showed that each ecotype had a different root phenotype and that under L-glutamate exposure, the primary root growth was inhibited, which was confirmed by quantitative data

from primary root length measurements. When the gene expression of all the isoforms of the *AtGLR2.5* gene and the splicing variant *AtGLR2.5c* were measured, a difference was shown between the ecotypes. The primer pairs amplified the whole *AtGLR2.5* gene, which means that this difference could have been due to any of the four splicing variants which could have been different for each ecotype. For each ecotype a correlation was seen between the gene expression of the all the isoforms of the *AtGLR2.5* gene and the primary root growth. C24 had the smallest root phenotype and the lowest gene expression for all the isoforms of the *AtGLR2.5* gene followed by NW and Col-8. So there could be a link between root phenotype and the expression pattern.

The gene expression for one of the splicing variants: the *AtGLR2.5c* splicing variant was measured, because as previously mentioned, the transformation with the *AtGLR2.5c* cDNA was sufficient to restore L-glutamate sensitivity in *AtGLR2.5* mutants (unpublished data), and was up-regulated under each treatment. Under each treatment the primary root growth was inhibited which could lead to a correlation between splicing variant *AtGLR2.5c* and the primary root inhibition. This could suggest that the splicing variant *AtGLR2.5c* induces the primary root growth inhibition because for instance for the 1 mM L-Glu concentration used for the inhibition of primary root growth in Col-8 had a higher gene expression than the 50 µM L-Glu concentration.

To get more insight in the involvement of the other splicing variants of the *AtGLR2.5* gene in root development under L-glutamate stress, this experiment was repeated with the same ecotypes, except the ecotype Col-8 was replaced by the ecotype Col-0.

Phenotypic screening and primary root length. Five-day-old seedlings of 3 different ecotypes; NW, C24 and Col-0 were transferred to treatment plates for one week which contained 0, 50 µM and 1 mM L-Glu. C24 had still the shorter root phenotype under normal growth conditions. After the transfer to treatment plates, the primary roots were only slightly or not inhibited. But still the later roots increased in number and length (Fig. 8).

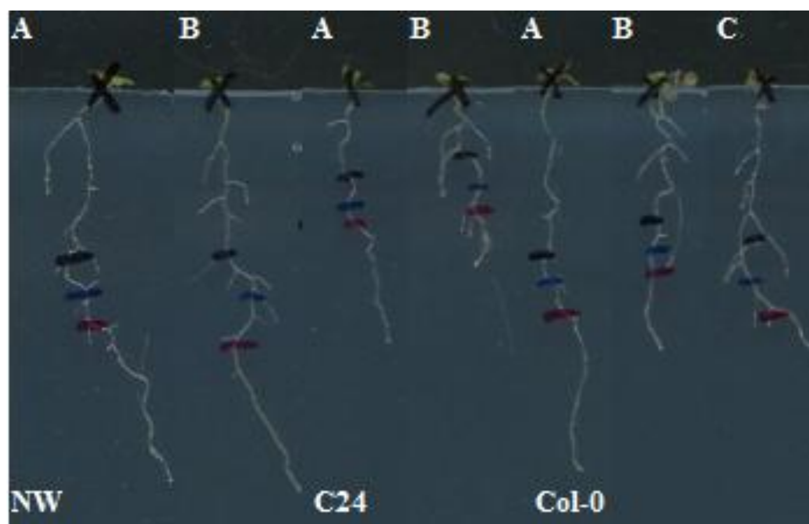


Figure 8: Effect of L-Glu on root phenotype of different eleven-day-old ecotypes of *A. thaliana*. The 3 different wild-type ecotypes; NW, C24 and Col-0 were transferred to treatment plates after five days which contained solid 50 X diluted Gamborg's B5 macro- and micronutrients medium; only medium (A), 50 µM (B) and 1 mM (C) L-Glu. On day zero, one and two a mark was set on the vertical agar plate.

Quantitative data obtained from measuring the primary root growth from seedlings after transfer to treatment medium (Fig. 9), confirmed the visual observations made out the root phenotypes showed in figure 8. Except for Col-0, where the quantitative data showed a difference in primary root length for the 50 µM L-Glu and 1 mM L-Glu.

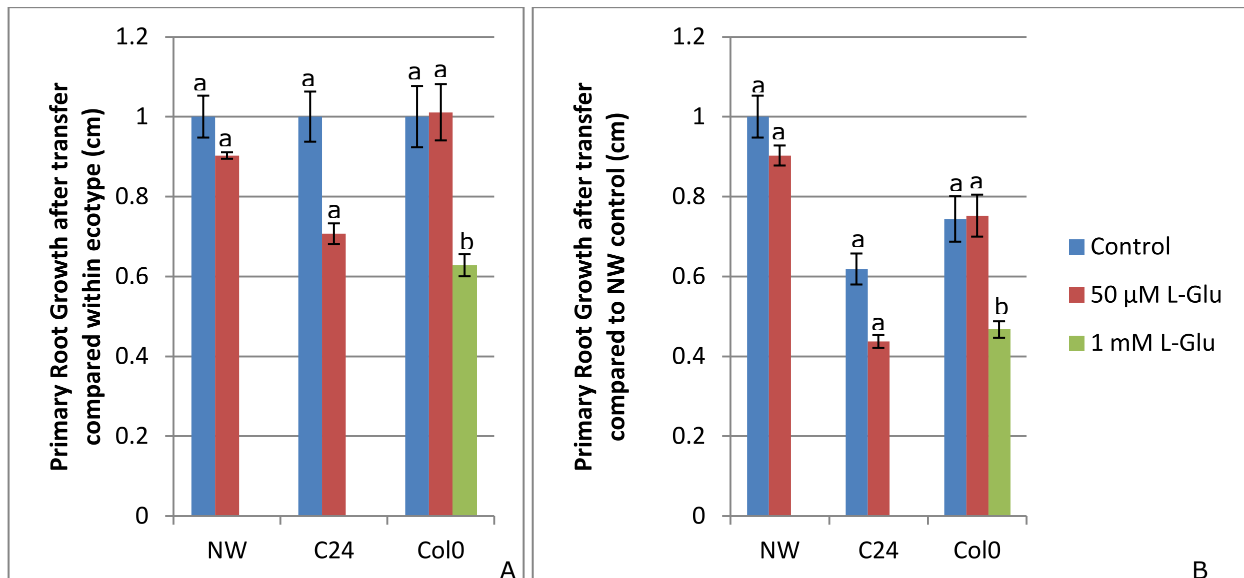


Figure 9: Measurement of primary root growth inhibition in different ecotypes of *A. thaliana* exposed to L-Glu. The primary root growth after transfer to treatment media for one week (in cm) was shown in function of the different L-glutamate concentrations from different eleven-day-old ecotypes of *A. thaliana* exposed to 0, 50 µM and 1 mM L-Glu, compared within ecotype (A) and compared to NW control (B). Different letters indicate statistically significant differences within ecotype (a, b)($P < 0.05$; two-way ANOVA and Tukey's HSD test). For each treatment, n = 10.

Statistical analysis using a 'two-way ANOVA', showed that the treatment with L-glutamate did not have a significant reduction in primary root growth compared to the control within each ecotype. Only for 1 mM L-Glu treatment a significant reduction in primary root growth was observed. When compared to the control conditions of NW, the results were unchanged and primary root growth was slightly inhibited for each ecotype under L-glutamate stress except for the 50 µM L-Glu treatment for Col-0.

Quantitative gene expression analysis. It was attempted to quantify the expression of all the different splicing variants by a qRT-PCR. qPCR primers specific for each isoform were designed (Appendix 1). The gene expression of all the isoforms and all the different splicing variants; *AtGLR2.5a*, *AtGLR2.5b*, *AtGLR2.5c* and *AtGLR2.5d* of the *AtGLR2.5* gene were measured. This to investigate if there was a difference between ecotypes for the other splicing variants as well and if L-glutamate had a difference on the gene expression of those splicing variants. However, data is only shown for all the isoforms and splicing variant *AtGLR2.5b* and *AtGLR2.5c.* The splicing variants *AtGLR2.5a* and *AtGLR2.5d* had a low expression as noticed by the high Ct values, which also made it difficult to measure primer efficiency.

In this experiment the expression of all the isoforms of the *AtGLR2.5* gene was clearly down-regulated under L-glutamate exposure for the C24 and Col-0 ecotypes compared to the control conditions but not for the NW ecotype(Fig. 10-A). When compared to the NW control conditions, the expression of all the isoforms of the *AtGLR2.5* gene differs between each ecotype, where C24 has now the highest expression and NW and Col-8 have a similar expression. But all the isoforms of the *AtGLR2.5* gene were

still down-regulated for the 50 µM L-Glu treatment for C24 and Col0 (Fig. 10-B). No statistically differences were observed.

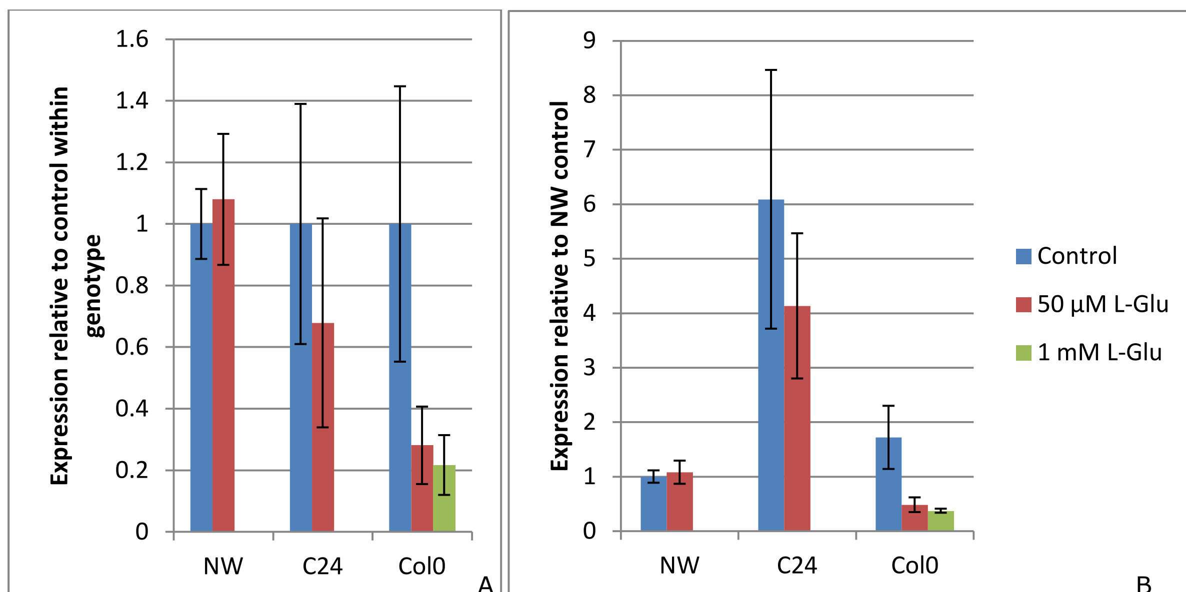


Figure 10: RT-qPCR based gene expression analysis of all the isoforms of *AtGLR2.5* gene in *A. thaliana* under L-Glu treatment. This figure shows the gene expression of all the isoforms of *AtGLR2.5* gene relative to the control within each ecotype (A) and relative to the control of the NW wild-type ecotype (B) when exposed to L-Glu. A two-way ANOVA and Tukey's HSD test was performed with no statistically significant differences ($P < 0.05$).For each ecotype, n = 5.

The gene expression of the splicing variant *AtGLR2.5b* was up-regulated for NW and C24 under 50 µM L-Glu exposure and was down-regulated for Col-0 under both different L-Glu treatments (Fig. 11-A). When the gene expression was compared to the control of NW, the gene expression of the splicing variant *AtGLR2.5b* differed in each ecotype with the highest expression in C24 (Fig. 11-B). No statistically differences were observed.

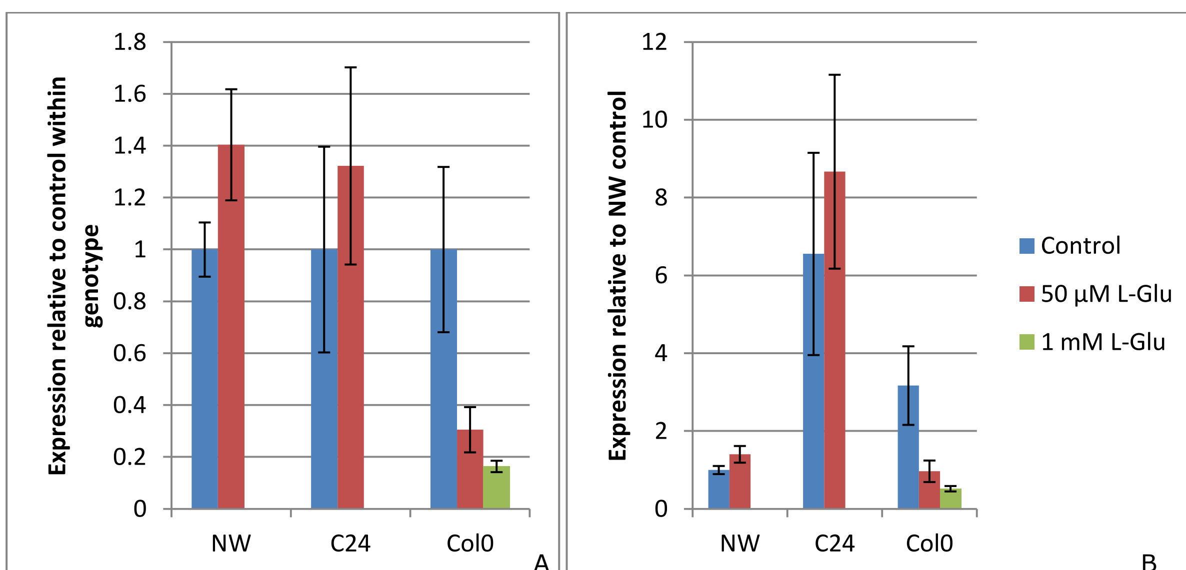


Figure 11: RT-qPCR based gene expression analysis of the splicing variant *AtGLR2.5b* in *A. thaliana* under L-Glu treatment. This figure shows the gene expression of the splicing variant *AtGLR2.5b* relative to the control within each ecotype (A) and relative to the control of the NW wild-type ecotype (B) when exposed to L-Glu. A two-way ANOVA and Tukey's HSD test was performed with no statistically significant differences ($P < 0.05$). For each ecotype, n = 5.

The gene expression of the splicing variant *AtGLR2.5c* was down-regulated under 50 μM L-Glu for C24 but up-regulated for NW and Col0. For the 1 mM L-Glu treatment, Col-0 was slightly up-regulated (Fig. 12-A). When the gene expression was compared to the control of NW, the gene expression of the splicing variant *AtGLR2.5c* differed between ecotypes with the highest expression in C24 (Fig.12-B). No statistically differences were observed.

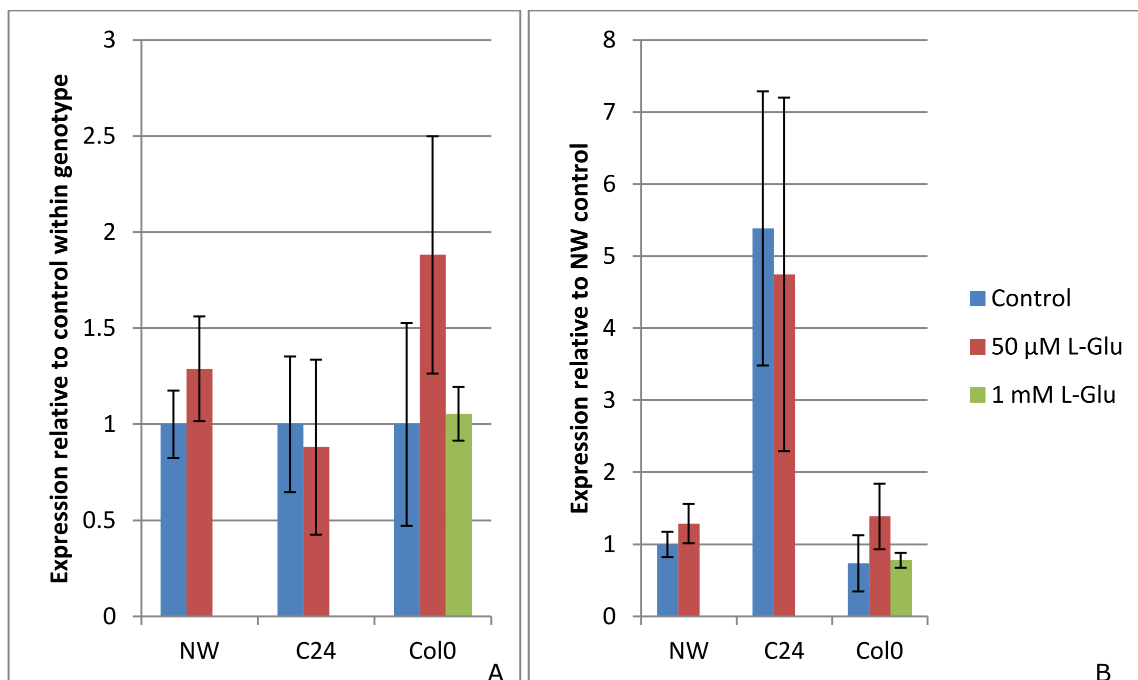


Figure 12: RT-qPCR based gene expression analysis of the splicing variant *AtGLR2.5c* in *A. thaliana* under L-Glu treatment. This figure shows the gene expression of the splicing variant *AtGLR2.5c* relative to the control within each ecotype (A) and relative to the control of the NW wild-type ecotype (B) when exposed to L-Glu. A two-way ANOVA and Tukey's HSD test was performed with no statistically significant differences ($P < 0.05$). For each ecotype, n = 5.

The phenotypic screening showed that each ecotype had a different root phenotype and that under L-glutamate exposure, the primary root growth should be inhibited. This phenomenon was not clearly seen in this experiment and not confirmed by the quantitative data from the primary root length measurements, except for Col-0 under 1 mM L-Glu exposure. When the gene expression of all the isoforms of the *AtGLR2.5* gene and the splicing variants: *AtGLR2.5b* and *AtGLR2.5c* were measured, a difference was shown between the ecotypes. For all the isoforms, this difference can be due to any of the four splicing variants and only NW showed up regulation for all the isoforms. For the *AtGLR2.5b* splicing variant, the gene expression was up-regulated for NW and C24, and down-regulated for Col-0. This could be an indication that the splicing variant *AtGLr2.5b* is affected by L-glutamate treatment but more research should be performed if this splicing variant is involved in the primary root growth inhibition. The gene expression for the splicing variant *AtGLR2.5c* was also measured and was up-regulated for NW and Col-0 and down-regulated in C24 under 50 μM L-Glu treatment. The 1 mM L-Glu treatment in Col-0 did not show any effect of L-Glu on the gene expression of splicing variant *AtGLR2.5c*. When these results were compared to the previous obtained results, a difference was seen. For NW and C24 the gene expression for the splicing variant *AtGLR2.5c* was up-regulated and a significant reduction in primary root growth was seen in the first experiment which could be an indication for the involvement of this splicing variant in primary root growth. The second experiment did

not have this significant primary root growth inhibition and clear up regulation of the gene expression, so maybe there is no correlation between the two parameters. The Col-0 ecotype had a strong significant inhibition in primary root growth under 1 mM L-Glu in both experiments but a different gene expression for the splicing variant *AtGLR2.54c*, so this could be an indication that the hypothesis that the splicing variant *AtGLR2.5c* is involved in primary root growth under L-glutamate exposure is not true.

3.2 L-glutamate and metal sensitivity in Col-0

This part of the research investigated if L-glutamate and different metals give a distinct root phenotype and had a different effect on primary root length in the Col-0 wild-type of *A. thaliana*. Also whether there was an influence of L-glutamate and metals on alternative splicing and gene expression of the *AtGLR2.5* gene and if this differed between the treatments.

Phenotypic screening and primary root length. For this part of the experiment, five-day-old seedlings of Col-0 wild-type *A. thaliana* were transferred to treatment plates for one week which contained; 0, 1 mM L-Glu, 5 µM Cd, 10 µM Cu and 75 µM Zn.

The root phenotype of Col-0 under the different metal- and glutamate treatments in vertical agar plate systems had some similar morphological outcomes. The seedlings under L-glutamate treatment (Fig. 13-B) had longer and more lateral roots compared to the seedlings on the metal treatment medium (Fig. 13C-E). This, as previous mentioned, was because the lateral roots gain glutamate sensitivity later in their development than the primary roots (9). The three metals used in this experiment have different properties in plants where Cd is non-essential and non-redox active, Zn and Cu are essential but are non-redox and redox-active respectively. But as mentioned above they also, like L-glutamate, inhibited the primary root growth. The lateral roots were more inhibited by Cu and Cd than by L-glutamate at the concentration that caused similar inhibition of the primary root growth. Zn on the contrary had a complete systemic effect on the primary and lateral root growth (Fig. 13-E)(5).

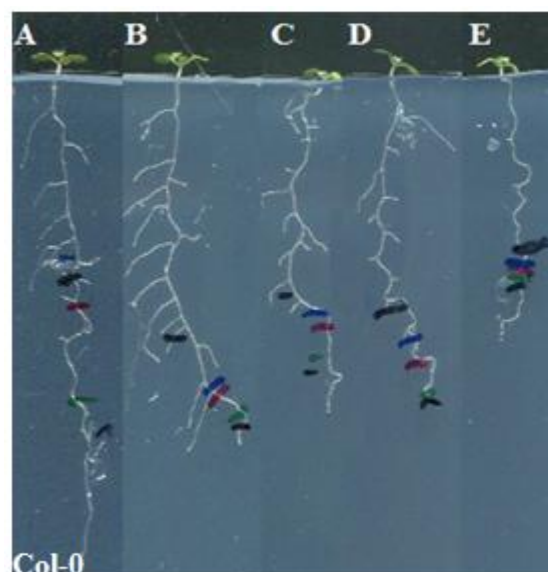


Figure 13: Effect of L-Glu, Cd, Cu and Zn on root phenotype of eleven-day-old Col-0 wild-type *A. thaliana*. The Col-0 wild-type ecotype was transferred to treatment plates after five days which contained solid 50 X diluted Gamborg's B5 macro- and micronutrients medium; only medium (A), 1 mM L-Glu (B), 5 µM Cd (C), 10 µM Cu (D) and 75 µM Zn (E). On day zero, one, two, four and five a mark was set on the vertical agar plate.

Quantitative data (Fig. 14), obtained from measuring the primary root growth from seedlings after transfer to treatment medium, confirmed the visual observations made out the root phenotypes shown in figure 13.

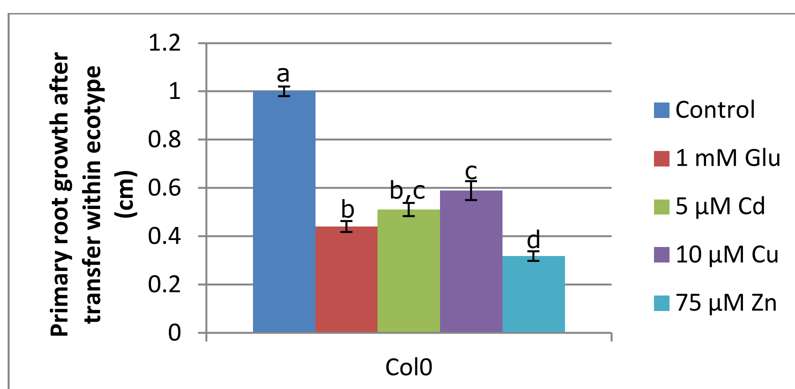


Figure 14: Measurement of primary root growth inhibition in the Col-0 wild-type ecotype of *A. thaliana* exposed to L-Glu, Cd, Cu and Zn. The primary root growth after transfer to treatment media for one week (in cm) was shown in function of the different treatments; 1mM L-Glu, 5 µM Cd, 10 µM Cu and 75 µM Zn from eleven-day-old Col-0 wild-type ecotype of *A. thaliana*. Different letters indicate statistically significant differences between treatments(a, b, c, d)($P < 0.05$; one-way ANOVA and Tukey's HSD test). For each treatment n = 20.

Statistical analysis using a 'two-way ANOVA' showed that each treatment had a significant reduction in primary root growth compared to the control condition. Where the Cd treatment had no significant differences with L-Glu and Cu treatment.

Expression of the different splicing variants of *AtGLR2.5*. To check which splicing variants of the whole *AtGLR2.5* gene were expressed in Col-0 under the different treatments and to investigate whether there was a difference in splicing variants between treatments, a RT-PCR analysis was performed for this experiment with two different primer pairs designed from the first- (Fig. 14-1) and the second (Fig. 14-2) putative startcodon. Both primers pairs were designed to amplify the whole *AtGLR2.5* gene and to obtain the four different splicing variants.

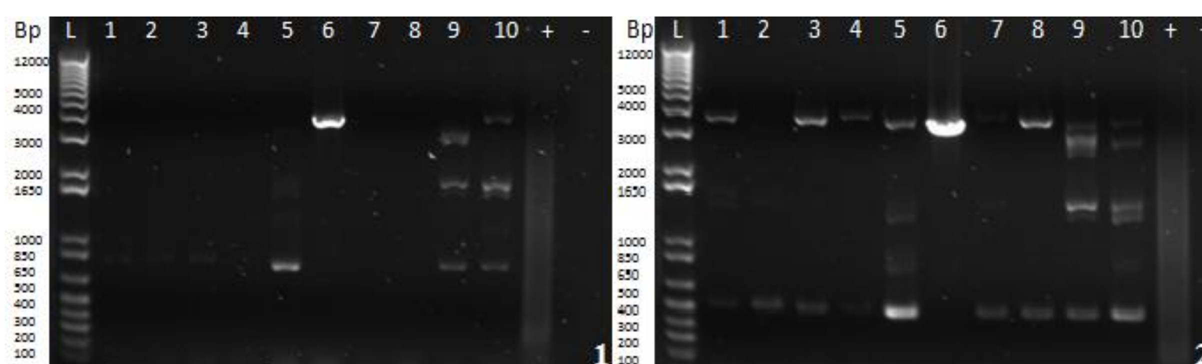


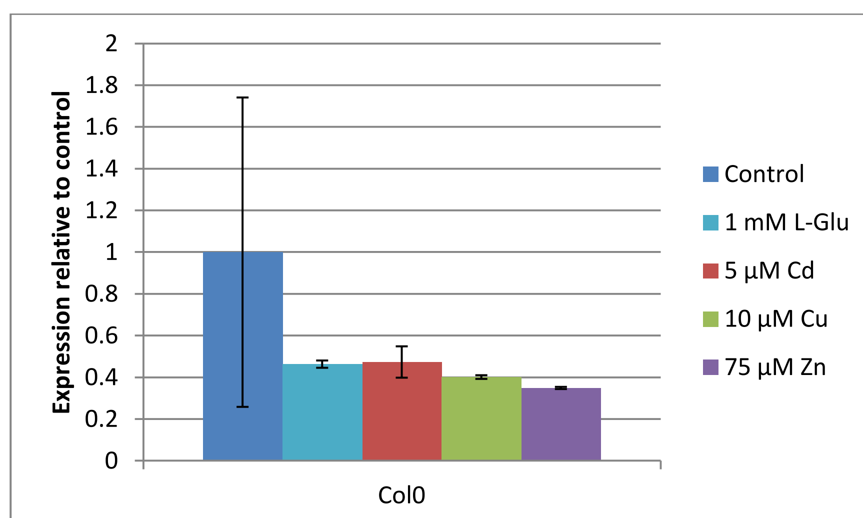
Figure 15: RT-PCR analysis of the splicing variants of the *AtGLR2.5* gene under L-Glu, Cd, Cu and Zn treatment of the Col-0 wild-type ecotype of *A. thaliana*. *AtGLR2.5* gene cDNA was amplified, using two specific primer pairs from the first- (Fig.8-1) and the second (Fig.8-2) putative startcodon. Duplicate samples: Col-0 grown on control (lane 1, 6), 1 mM L-Glu (lane 2, 7), 5 µM Cd (lane 3, 8), 10 µM Cu (lane 4, 9) and 75 µM Zn medium (lane 5, 10). Also a positive control for successful RT (+), contained human GADPH control RNA template and GAPDH-specific PCR primers, and a negative control(-), where cDNA template was replaced by RNase-free water, were analysed.

Like previously described, the splicing variants were identified by a 1 Kb plus ladder and differed in size between both gels. In figure 15-2, the splicing variants were 205 bp longer than shown in figure 15-1.

The data obtained from this RT-PCR differed between duplicates which can be an indication of different RNA input or efficiency of cDNA synthesis, yielding variable cDNA input in the amplification reaction. There is a possibility that the splicing variants differed between the treatments and some splicing variants were expressed more than other splicing variants. This could be an indication that each treatment had a specific influence on the expression and/or alternative splicing of the *AtGLR2.5* gene. But to get a more confident quantification of the different splicing variants, a qRT-PCR should be performed with normalisation.

Quantitative gene expression analysis. The quantification of the different splicing variants was performed by a qRT-PCR. The gene expression of all the isoforms and the splicing variant *AtGLR2.5c* of the *AtGLR2.5* gene were measured. This to investigate if there was an influence of the different treatments: 1 mM L-Glu, 5 µM Cd, 10 µM Cu and 75 µM Zn on gene expression of all the splicing variants of the *AtGLR2.5* gene in general, and the splicing variant *AtGLR2.5c* specifically.

The expression of all the isoforms of the *AtGLR2.5* gene was clearly down-regulated under the different treatments compared to the control conditions. In the previous experiment, L-glutamate induced an increase in the gene expression of the *AtGLR2.5* gene at a 50 µM concentration, but for the Col-8 ecotype under 1 mM L-Glu exposure the *AtGLR2.5* gene was also down-regulated, this could suggest that a lower concentration of L-glutamate has an increasing effect on the gene expression and a high concentration a decreasing effect. The down-regulated effect is quite similar between the different treatments (Fig. 16). No statistically differences were observed.



Figur 16: RT-qPCR based gene expression analysis of all the isoforms of the *AtGLR2.5* gene under L-Glu, Cd, Cu and Zn treatment of the Col-0 wild-type ecotype of *A. thaliana*. This figure shows the gene expression of all the isoforms of *AtGLR2.5* gene relative to the control when Col-0 was exposed to 1 mM L-Glu, 5 µM Cd, 10 µM Cu and 75 µM Zn. A one-way ANOVA and Tukey's HSD test was performed with no statistically significant differences ($P < 0.05$). For each treatment, n = 2.

The expression of the splicing variant *AtGLR2.5c* was also investigated and each treatment had an increase in gene expression for the splicing variant *AtGLR2.5c*. The 1 mM L-Glu, 5 µM Cd and 10 µM Cu had a similar gene expression for the splicing variant *AtGLR2.5c*. The gene expression increased significantly in two-fold under zinc exposure (Fig. 17).

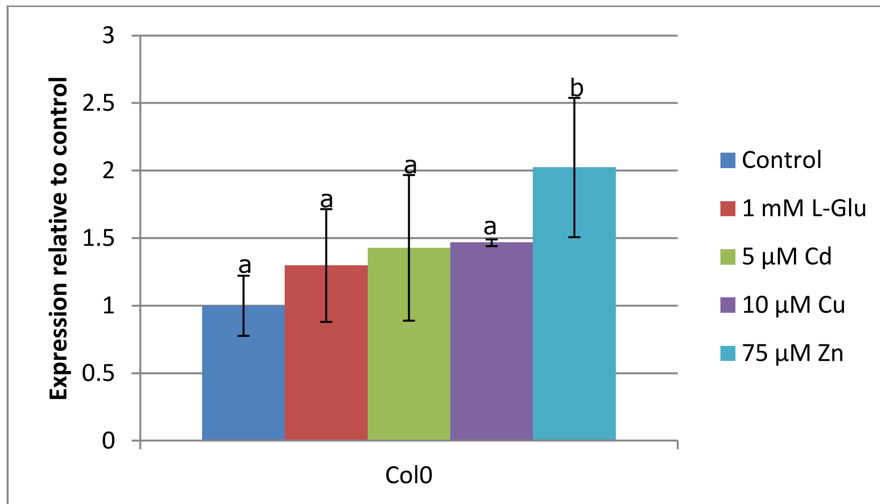


Figure 17: RT-qPCR based gene expression analysis of the splicing variant *AtGLR2.5c* under L-Glu, Cd, Cu and Zn treatment of the Col-0 wild-type ecotype of *A. thaliana*. This figure shows the gene expression of the splicing variant *AtGLR2.5c* relative to the control when Col-0 was exposed to 1 mM L-Glu, 5 µM Cd, 10 µM Cu and 75 µM Zn. Different letters indicate statistically significant differences between treatments(a, b)($P < 0.05$; one-way ANOVA and Tukey's HSD test). For each treatment, n = 5.

The phenotypic screening showed that each treatment induced different root phenotypes, but under each treatment primary root growth was inhibited. Under L-glutamate exposure, the root phenotype had more lateral roots, where under zinc treatment, a complete systemic effect on root primary and lateral root growth occurred. Copper and cadmium had a more similar root phenotype. These outcomes were confirmed by quantitative data from primary root measurements. The gene expression of all the isoforms of the *AtGLR2.5* gene was down-regulated under each treatment, even under the L-glutamate treatment which was up-regulated for the experiment investigating the effect of L-glutamate of ecotypes. This can be due to the high concentration of 1 mM L-Glu whereas in the other experiment about the effect of L-glutamate 50 µM L-Glu was used, except for the Col-8 ecotype, which was also exposed to 1 mM L-Glu and showed down-regulation of the *AtGLR2.5* gene under this condition. So this could indicate that the used concentration of L-glutamate has an influence on the gene expression of all the isoforms of the *AtGLR2.5* gene. The splicing variant *AtGLR2.5c* was also measured, which was up-regulated under each treatment, with the highest expression for the zinc treatment. Out of these results there could be a correlation between the complete systemic effect of the zinc treatment and up-regulated gene expression of the splicing variant *AtGLR2.5c*. The differences in gene expression for the splicing variant *AtGLR2.5c* could be an indication for the differences in root phenotype under each treatment and would suggest that the second hypothesis is true and that there was an involvement of the splicing variant in the formation of the inhibited root phenotype.

3.3 Glutamate sensitivity of MAPK signalling mutants

This experiment investigated the effect of L-glutamate on primary root growth in different mutants with a mutation in *MKK*-related genes. This to get more insight in the involvement of different MAPK-pathways in root development under L-glutamate stress.

Phenotypic screening and primary root length. The five-day-old seedlings of 8 different MAPK signalling mutants; *mkk1*, *mkk2*, *mkk7*, *mkk9*, *mekk1/2/3*, *mkk10*, *mkk3* and *mkk2/3* were transferred to treatment plates for one week which contained 0 and 2 mM L-Glu. The Col-0 ecotype was used as control.

Each of the eight different MAPK signalling mutants had its own distinct phenotype in the vertical agar system that arose from the deletion in the MAPK-pathway. For example *mkk7* and *mkk9* (Fig. 18) had a similar primary root growth but shorter lateral roots than compared to the control wild-type Col-0. The other mutants; *mkk1*, *mkk2*, *mkk1/2/3*, *mkk10*, *mkk3* and *mkk2/3* (Fig. 18 and 19) had a more similar primary root- and lateral root growth. When the five-day-old seedlings of each MAPK signalling mutant were transferred to the treatment medium, which contained 2 mM L-Glu, the primary roots of all MAPK signalling mutants were inhibited, the lateral roots on the other hand increased in number and length, due to the fact that lateral roots are not immediately inhibited by the presence of glutamate, they gain the glutamate sensitivity later in their development than primary roots (9). Previous research showed that the triple mutant *mkk1/2/3* was almost insensitive to L-glutamate and furthermore the effect on primary root architecture was absent in this mutant (21). But this effect of insensitivity was not shown in the obtained results in this experiment (Fig. 18), this could be due to other factors who could have an influence on root development like; humidity, temperature, light intensity, growth medium and aeration (25). That same research showed an unaltered L-glutamate sensitivity for *mkk2* and *mkk3* (Fig. 17 and 18), which was observed in this experiment as well.

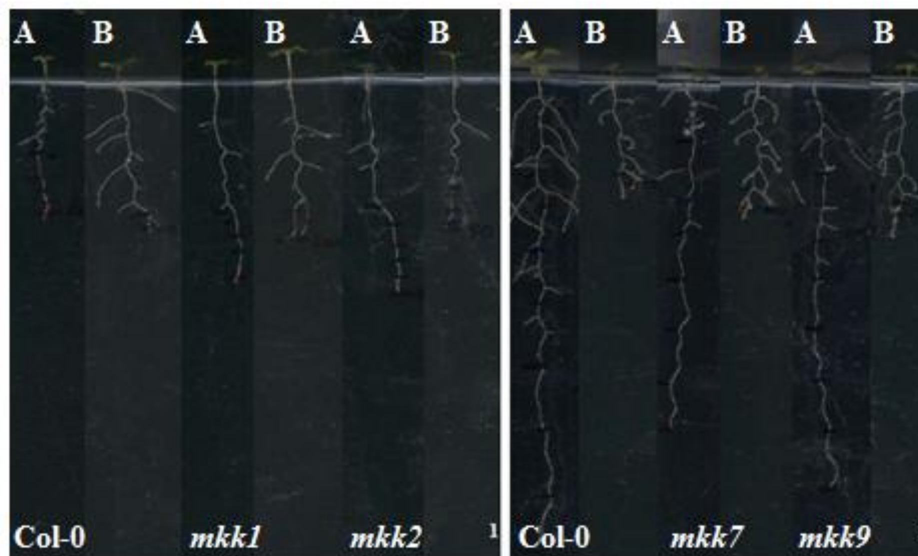


Figure 18: Effect of L-Glu on root phenotype of different eleven-day-old MAPK signalling mutants of *A. thaliana*. The 4 different *MKK*-mutants; *mkk1*, *mkk2*, *mkk7* and *mkk9* were transferred to treatment plates after five days which contained solid 50 X diluted Gamborg's B5 macro- and micronutrients medium; only medium (A) and 2 mM (B) L-Glu. Each mutant was compared to Col-0. Each day during the one-week-treatment, a mark was set on the vertical agar plate.

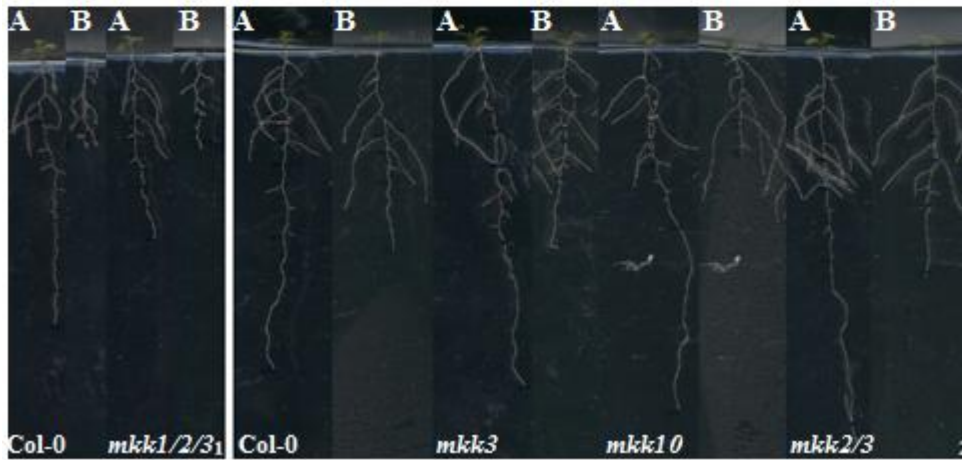


Figure 19: Effect of L-Glu on root phenotype of different eleven-day-old MAPK signalling mutants of *A. thaliana*. The 4 different *MKK*-mutants; *mkk1/2/3*, *mkk10*, *mkk3* and *mkk2/3* were transferred to treatment plates after five days which contained solid 50 X diluted Gamborg's B5 macro- and micronutrients medium; only medium (A) and 2 mM (B) L-Glu. Each mutant was compared to Col-0. Each day during the one-week-treatment, a mark was set on the vertical agar plate.

Quantitative data (Fig. 20), obtained from measuring the primary root growth from seedlings after transfer to treatment medium, confirmed the visual observations made out the root phenotypes shown in figures 18 and 19. The triple mutant *mkk1/2/3* had a longer primary root compared to the Col-0 under L-glutamate exposure. So this could indicate that indeed, this mutant is less sensitive to L-glutamate like previously proven.

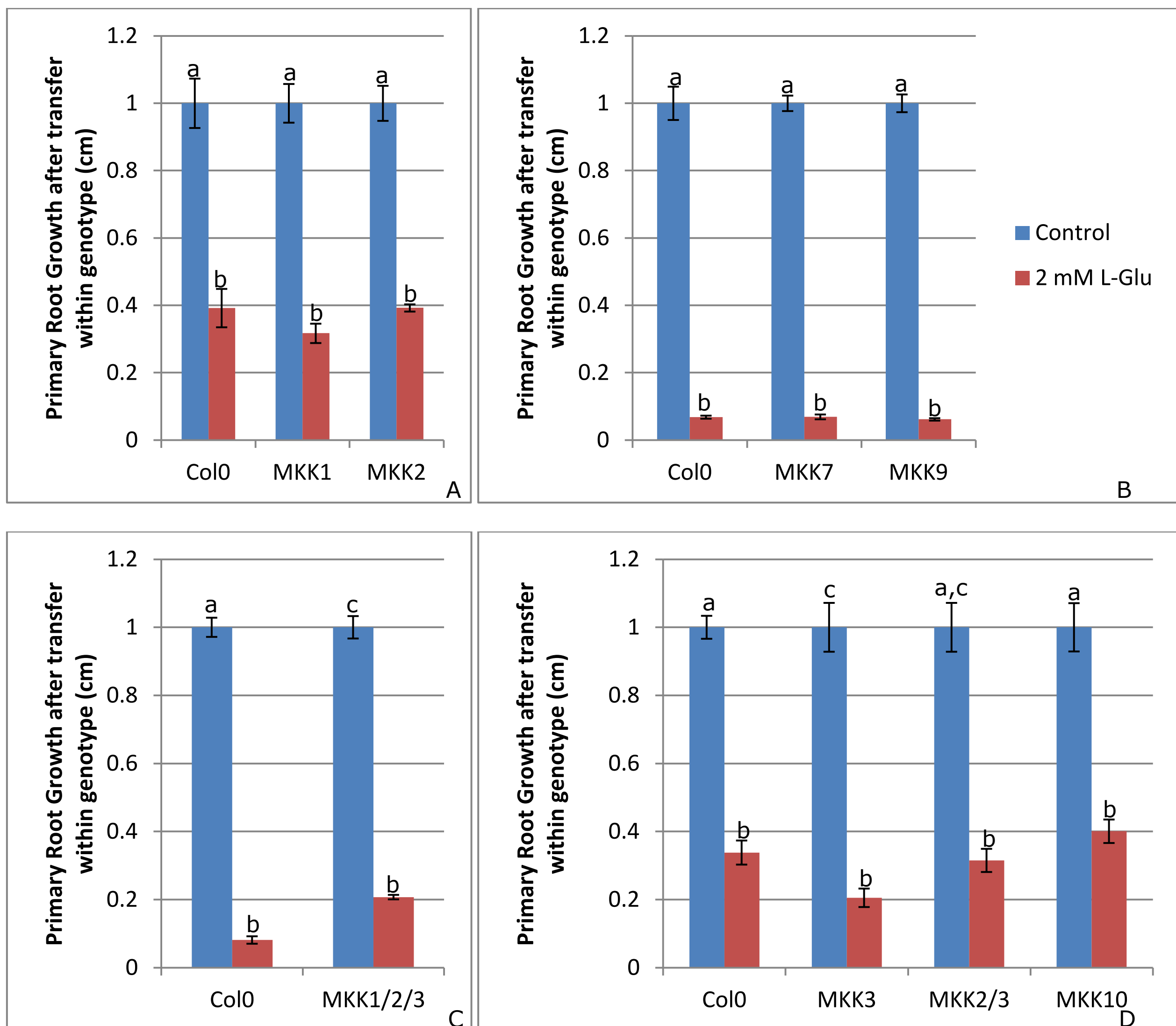


Figure 20: Measurement of primary root growth inhibition of different eleven-day-old MAPK signalling mutants of *A. thaliana* exposed to L-Glu. The primary root growth after transfer to treatment media (in cm) was shown in function of the different L-glutamate concentrations from different twelve-day-old MAPK signalling mutants of *A. thaliana* exposed to 0 and 2 mM L-Glu. Different letters indicate statistically significant differences within MAPK signalling mutants (a, b, c)($P < 0.05$; two-way ANOVA and Tukey's HSD test).For Col-0, *mkk1*, *mkk2*, *mkk9*, *mkk1/2/3*, *mkk2/3* and *mkk10*; n = 15, for *mkk7*; n = 8 and for *mkk3*; n = 11.

Statistical analysis using a 'two-way ANOVA' showed that the treatment of each MAPK signalling mutant with 2 mM L-Glu had a significant reduction in primary root growth compared to the control conditions This could be an indication that each of the different MKKs was not involved in the used MAPK pathway under L-glutamate exposure. The fact that each MAPK signalling mutant had a similar primary root length compared to the Col-0 wild-type under L-glutamate exposure indicates that only the triple mutant was less sensitive to L-glutamate and was involved in the signalling pathway.

4 Conclusion & Synthesis

This research project investigated the mechanism of primary root growth inhibition under L-glutamate, copper, cadmium and zinc stress. Previous research showed that those stresses induce primary root growth inhibition and induce lateral root growth but the genes and pathways involved in this process are still unclear.

A possible gene that could be involved in this process belongs to the family of glutamate receptor-like proteins: the *AtGLR2.5* gene. previous research showed that this gene could restore the sensitivity to L-glutamate. This gene has four different splicing variants; *AtGLR2.5a*, *AtGLR2.5b*, *AtGLR2.5c* and *AtGLR2.5d*, all of which encode incomplete GLR proteins. Only the splicing variant *AtGLr2.5c* is previously investigated and could restore L-glutamate sensitivity. This led to the first hypothesis, that this splicing variant is involved in the primary root growth inhibition under L-glutamate stress. Comparing the results from the phenotypic screening and gene expression from the first experiment showed that there could be a link between inhibition of the primary root growth and the expression of the splicing variant *AtGLR2.5c.* By performing a second similar experiment, where the other splicing variants were tested, showed that only *AtGLR2.5b* and *AtGLR2.5c* could be involved. In one ecotype: Col-0, the primary root growth was inhibited and the gene expression of splicing variant *AtGLR2.5c* was unaffected by L–glutamate so the first hypothesis is not true and more research should be performed.

A similar root growth inhibition was seen in previous research where plants grew on treatment plates containing metals like copper, cadmium and zinc. The fact that those stresses induced a similar root phenotype like L-glutamate, a second hypothesis was formed, which stated that the inhibition in de the primary root growth was also due to the splicing variant *AtGLR2.5c.* The results showed a positive correlation between the primary root growth inhibition and the gene expression of the splicing variant *AtGLR2.5c* for each treatment. The zinc treatment had a complete systemic effect on the seedling and had the highest gene expression for the splicing variant *AtGLR2.5c*. These observations suggest that the second hypothesis is true and that this splicing variant is involved in het primary root growth inhibition seen under metal stress.

The side experiment to test whether the different MAPK signalling mutants where involved in the MAPK signalling under L-glutamate stress showed that none of them was involved except maybe the triple mutant: *mkk1/2/3.*

Further research should be performed on the different splicing variants of the *AtGLR2.5* gene and their involved under L-glutamate stress, also if one of them is involved in the primary root growth inhibition under metal stress. The different MAPK signalling mutants where not involved in the signalling under L-glutamate but maybe they are involved in the signalling under metal stress, further investigation should be performed to get more insights in the complex mechanism of primary root growth.

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6 Appendix

6.1 Appendix 1: the whole sequence of the *AtGLR2.5* gene and designed primers

The sequence of the *AtGLR2.5* gene was obtained from the TAIR database (www.arabidopsis.org) with introns and exons. This sequence was used to design the different primer pairs used during the experiments:

Primer whole genome:	GLR2.5whole-F1	ATGTCTCTTTTCCATCATCTC
	GLR2.5whole-F2	ATGGCTTCAAGACAAGGATTG
	GLR2.5whole-R	CTAGAGTTTAGGTTTGACTAT
Primer 4 varianten:	GLR2.5allisof-F	TGCTTCACCCAATCTACCAA
	GLR2.5allisof-R	CAAAGCTGTGTCGACTTCCA
Primer a:	GLR2.5A-F	TCGGGTCAAGACTCTTTTCG
	GLR2.5A-R	CAAGCTTGATTCCCCCATTA
Primer b:	GLR2.5B-F	TTAATGGTGGCAGCAGAAGA
	GLR2.5B-R	TTCACAAGTTTGGTGCGTACA
Primer c:	GLR2.5C-F	CGTTCGATGAGGTCGCTTAT
	GLR2.5C-R	GTGGAAATGCAAAGCCAAAG
Primer d:	GLR2.5D-F	ATGGCTTCAAGACAAGGATTG
	GLR2.5D-R	TCTGACACCAATGGAGATCCT

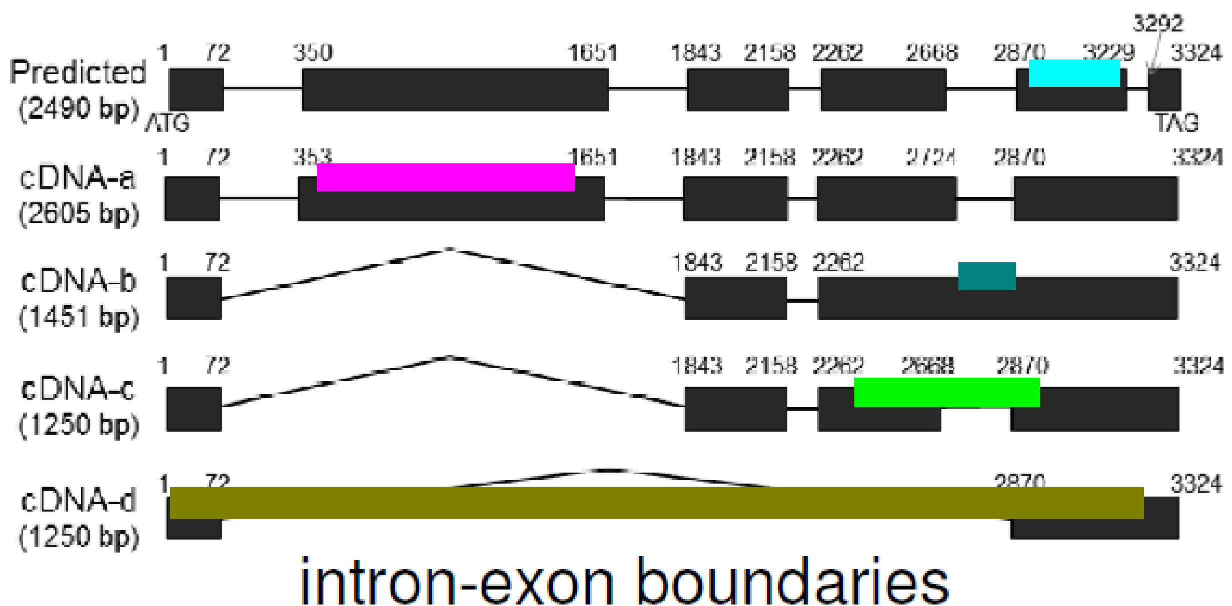

Figure 21: Schematic overview of the different splicing variants of the *AtGLR2.5* gene with their unique primer sequence localisation. The length of the amplicons is not shown in this figure.

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***************M**A**S**R**Q**G**L**S**S**T**S**E**T**P**N**K**L**L**L**V**L**P**L**Q*******************************
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*E**V**V**P**I**Y**V**D**N**E**F**G**E**G**I**L**P**N**L**V**D**A**F**Q**E**I**N**V**R**I**R**Y**R**S**A**I**S**L**H
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YS**D**D**Q**I**K**K**E**L**Y**K**L**M**T**M**P**T**R**V**F**I**V**H**M**L**P**D**L**G**S**R**L**F**S**I**A**K**

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TGCAATGTCAGTTGAGGAAATTAGGCACGTAAACATGAGTTTCAACACGACCAAAGAAGACACTTCAAGAGATGATATTGGGACTGATCTTGATGAACTCGGCGTTGCTCTATCTG
TGCAATGTCAGTTGAGGAAATTAGGCACGTAAACATGAGTTTCAACACGACCAAAGAAGACACTTCAAGAGATGATATTGGGACTGATCTTGATGAACTCGGCGTTGCTCTATCTG

AM**S**V**E**E**I**R**H**V**N**M**S**F**N**T**T**K**E**D**T**S**R**D**D**I**G**T**D**L**D**E**L**G**V**A**L**S**
AM**S**V**E**E**I**R**H**V**N**M**S**F**N**T**T**K**E**D**T**S**R**D**D**I**G**T**D**L**D**E**L**G**V**A**L**S**
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GTCCCAAGCTTCTTGATGCCTTGTCAACAGTCAGTTTCAAAGGTGTTGCCGGGAGATTTCAGCTAAAAAACGGAAAGCTAGAGGCGACGACTTTCAAGATTATCAATATAGAGGAA
GTCCCAAGCTTCTTGATGCCTTGTCAACAGTCAGTTTCAAAGGTGTTGCCGGGAGATTTCAGCTAAAAAACGGAAAGCTAGAGGCGACGACTTTCAAGATTATCAATATAGAGGAA

G**P**K**L**L**D**A**L**S**T**V**S**F**K**G**V**A**G**R**F**Q**L**K**N**G**K**L**E**A**T**T**F**K**I**I**N**I**E**E

G**P**K**L**L**D**A**L**S**T**V**S**F**K**G**V**A**G**R**F**Q**L**K**N**G**K**L**E**A**T**T**F**K**I**I**N**I**E**E
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AGCGGTGAAAGAACGGTTGGATTTTGGAAATCAAAAGTAGGATTAGTAAAGAGCTTAAGAGTAGATAAAGTGTCTCACAGCTCCCGTCGCCTTAGACCGATAATATGGCCTGGTGA
AGCGGTGAAAGAACGGTTGGATTTTGGAAATCAAAAGTAGGATTAGTAAAGAGCTTAAGAGTAGATAAAGTGTCTCACAGCTCCCGTCGCCTTAGACCGATAATATGGCCTGGTGA

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*S**G**E**R**T**V**G**F**W**K**S**K**V**G**L**V**K**S**L**R**V**D**K**V**S**H**S**S**R**R**L**R**P**I**I**W**P**G**D

*S**G**E**R**T**V**G**F**W**K**S**K**V**G**L**V**K**S**L**R**V**D**K**V**S**H**S**S**R**R**L**R**P**I**I**W**P**G**D
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CACTATTTTTGTGCCTAAAGGTTGGGAATTCCCAACAAACGCAAAGAAGCTGCGAATAGCAGTTCCAAAGAAGGATGGTTTCAACAATTTTGTTGAGGTAACCAAGGATGAAAATA
CACTATTTTTGTGCCTAAAGGTTGGGAATTCCCAACAAACGCAAAGAAGCTGCGAATAGCAGTTCCAAAGAAGGATGGTTTCAACAATTTTGTTGAGGTAACCAAGGATGAAAATA

TI**F**V**P**K**G**W**E**F**P**T**N**A**K**K**L**R**I**A**V**P**K**K**D**G**F**N**N**F**V**E**V**T**K**D**E**N**

TI**F**V**P**K**G**W**E**F**P**T**N**A**K**K**L**R**I**A**V**P**K**K**D**G**F**N**N**F**V**E**V**T**K**D**E**N**
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CTAATGTTCCAACGGTCACCGGGTTTTGCATAGATGTTTTCAACACGGTAATGAGCCAAATGCCATATGCTGTCTCCTATGAGTACATCCCCTTTGATACGCCTGATGGAAAACCT
CTAATGTTCCAACGGTCACCGGGTTTTGCATAGATGTTTTCAACACGGTAATGAGCCAAATGCCATATGCTGTCTCCTATGAGTACATCCCCTTTGATACGCCTGATGGAAAACCT

T**N**V**P**T**V**T**G**F**C**I**D**V**F**N**T**V**M**S**Q**M**P**Y**A**V**S**Y**E**Y**I**P**F**D**T**P**D**G**K**P
T**N**V**P**T**V**T**G**F**C**I**D**V**F**N**T**V**M**S**Q**M**P**Y**A**V**S**Y**E**Y**I**P**F**D**T**P**D**G**K**P
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CGTGGAAGTTACGATGAAATGGTTTATAATGTGTTTCTTGGGgtaagttactctgatccctaaccatttgaaaaaacaattcctcttttttttttttctttcttatatacatacat
CGTGGAAGTTACGATGAAATGGTTTATAATGTGTTTCTTGGG--

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*R**G**S**Y**D**E**M**V**Y**N**V**F**L**G***
*R**G**S**Y**D**E**M**V**Y**N**V**F**L**G***
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aatcatgagacctgtaaaagtttttttttgtttttgatagttaaggcgtctacaatagggtggagagccaatattgagctaaaatgttaatatcttttttacaaatgatgttgaca
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gGAGTTTGATGGAGCTGTAGGTGATACAACAATTTTGGCTAATCGGTCGCATTATGTTGATTTCGCGTTGCCATACTCGGAGACCGGAATTGTATTCCTTGTACCAGTCAAGGATG
-GAGTTTGATGGAGCTGTAGGTGATACAACAATTTTGGCTAATCGGTCGCATTATGTTGATTTCGCGTTGCCATACTCGGAGACCGGAATTGTATTCCTTGTACCAGTCAAGGATG
-GAGTTTGATGGAGCTGTAGGTGATACAACAATTTTGGCTAATCGGTCGCATTATGTTGATTTCGCGTTGCCATACTCGGAGACCGGAATTGTATTCCTTGTACCAGTCAAGGATG
-GAGTTTGATGGAGCTGTAGGTGATACAACAATTTTGGCTAATCGGTCGCATTATGTTGATTTCGCGTTGCCATACTCGGAGACCGGAATTGTATTCCTTGTACCAGTCAAGGATG

EF**D**G**A**V**G**D**T**T**I**L**A**N**R**S**H**Y**V**D**F**A**L**P**Y**S**E**T**G**I**V**F**L**V**P**V**K**D**
EF**D**G**A**V**G**D**T**T**I**L**A**N**R**S**H**Y**V**D**F**A**L**P**Y**S**E**T**G**I**V**F**L**V**P**V**K**D**
EF**D**G**A**V**G**D**T**T**I**L**A**N**R**S**H**Y**V**D**F**A**L**P**Y**S**E**T**G**I**V**F**L**V**P**V**K**D**
EF**D**G**A**V**G**D**T**T**I**L**A**N**R**S**H**Y**V**D**F**A**L**P**Y**S**E**T**G**I**V**F**L**V**P**V**K**D**
****

GGAAAGAAAAAGGAGAATGGGTCTTCTTAAAGCCTTTAACAAAGGAGCTATGGTTGGTCACTGCTGCTTCTTTTCTCTACATTGGAATCATGGTTTGGATTTTTGAGTACCAAGCA
GGAAAGAAAAAGGAGAATGGGTCTTCTTAAAGCCTTTAACAAAGGAGCTATGGTTGGTCACTGCTGCTTCTTTTCTCTACATTGGAATCATGGTTTGGATTTTTGAGTACCAAGCA
GGAAAGAAAAAGGAGAATGGGTCTTCTTAAAGCCTTTAACAAAGGAGCTATGGTTGGTCACTGCTGCTTCTTTTCTCTACATTGGAATCATGGTTTGGATTTTTGAGTACCAAGCA
GGAAAGAAAAAGGAGAATGGGTCTTCTTAAAGCCTTTAACAAAGGAGCTATGGTTGGTCACTGCTGCTTCTTTTCTCTACATTGGAATCATGGTTTGGATTTTTGAGTACCAAGCA

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G**K**E**K**G**E**W**V**F**L**K**P**L**T**K**E**L**W**L**V**T**A**A**S**F**L**Y**I**G**I**M**V**W**I**F**E**Y**Q**A
G**K**E**K**G**E**W**V**F**L**K**P**L**T**K**E**L**W**L**V**T**A**A**S**F**L**Y**I**G**I**M**V**W**I**F**E**Y**Q**A
G**K**E**K**G**E**W**V**F**L**K**P**L**T**K**E**L**W**L**V**T**A**A**S**F**L**Y**I**G**I**M**V**W**I**F**E**Y**Q**A
G**K**E**K**G**E**W**V**F**L**K**P**L**T**K**E**L**W**L**V**T**A**A**S**F**L**Y**I**G**I**M**V**W**I**F**E**Y**Q**A
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GATGAGGAGTTCAGGGAACAGATGATAATTGATAAAATATCTAGTGTGTTCTACTTCTCGTTTTCGACTCTCTTTTTCGCACACAgtgagttaatctctgtcacacacaacttcag
GATGAGGAGTTCAGGGAACAGATGATAATTGATAAAATATCTAGTGTGTTCTACTTCTCGTTTTCGACTCTCTTTTTCGCACACA-------------------------------
GATGAGGAGTTCAGGGAACAGATGATAATTGATAAAATATCTAGTGTGTTCTACTTCTCGTTTTCGACTCTCTTTTTCGCACACA-------------------------------
GATGAGGAGTTCAGGGAACAGATGATAATTGATAAAATATCTAGTGTGTTCTACTTCTCGTTTTCGACTCTCTTTTTCGCACACA-------------------------------
--------------------------------------------------------------------------------------------------------------------
*D**E**E**F**R**E**Q**M**I**I**D**K**I**S**S**V**F**Y**F**S**F**S**T**L**F**F**A**H*********************************
*D**E**E**F**R**E**Q**M**I**I**D**K**I**S**S**V**F**Y**F**S**F**S**T**L**F**F**A**H*********************************
*D**E**E**F**R**E**Q**M**I**I**D**K**I**S**S**V**F**Y**F**S**F**S**T**L**F**F**A**H*********************************
*D**E**E**F**R**E**Q**M**I**I**D**K**I**S**S**V**F**Y**F**S**F**S**T**L**F**F**A**H*********************************
********************************************************************************************************************

ctactaaccattaccaatttacttgaaaatctttgaaacaagaaaactgatctacaaacattttctttatagGGAGGCCATCAGAGAGCTTTTTTACAAGGGTTCTTGTTGTGGTT
------------------------------------------------------------------------GGAGGCCATCAGAGAGCTTTTTTACAAGGGTTCTTGTTGTGGTT
------------------------------------------------------------------------GGAGGCCATCAGAGAGCTTTTTTACAAGGGTTCTTGTTGTGGTT
------------------------------------------------------------------------GGAGGCCATCAGAGAGCTTTTTTACAAGGGTTCTTGTTGTGGTT
--------------------------------------------------------------------------------------------------------------------
************************************************************************R**R**P**S**E**S**F**F**T**R**V**L**V**V**V
************************************************************************R**R**P**S**E**S**F**F**T**R**V**L**V**V**V
************************************************************************R**R**P**S**E**S**F**F**T**R**V**L**V**V**V
************************************************************************R**R**P**S**E**S**F**F**T**R**V**L**V**V**V
********************************************************************************************************************

TGGTGCTTTGTGTTGCTAATTCTGACTCAGAGCTACACAGCAACACTGACATCGATGCTGACAGTTCAAGAGCTTCGACCAACAGTGAGACACATGGATGATTTGAGGAAGAGCGG
TGGTGCTTTGTGTTGCTAATTCTGACTCAGAGCTACACAGCAACACTGACATCGATGCTGACAGTTCAAGAGCTTCGACCAACAGTGAGACACATGGATGATTTGAGGAAGAGCGG
TGGTGCTTTGTGTTGCTAATTCTGACTCAGAGCTACACAGCAACACTGACATCGATGCTGACAGTTCAAGAGCTTCGACCAACAGTGAGACACATGGATGATTTGAGGAAGAGCGG
TGGTGCTTTGTGTTGCTAATTCTGACTCAGAGCTACACAGCAACACTGACATCGATGCTGACAGTTCAAGAGCTTCGACCAACAGTGAGACACATGGATGATTTGAGGAAGAGCGG
--------------------------------------------------------------------------------------------------------------------
 W**C**F**V**L**L**I**L**T**Q**S**Y**T**A**T**L**T**S**M**L**T**V**Q**E**L**R**P**T**V**R**H**M**D**D**L**R**K**S**G
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 W**C**F**V**L**L**I**L**T**Q**S**Y**T**A**T**L**T**S**M**L**T**V**Q**E**L**R**P**T**V**R**H**M**D**D**L**R**K**S**G
 W**C**F**V**L**L**I**L**T**Q**S**Y**T**A**T**L**T**S**M**L**T**V**Q**E**L**R**P**T**V**R**H**M**D**D**L**R**K**S**G
 W**C**F**V**L**L**I**L**T**Q**S**Y**T**A**T**L**T**S**M**L**T**V**Q**E**L**R**P**T**V**R**H**M**D**D**L**R**K**S**G
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AGTGAACATTGGATATCAAACTGGTTCGTTTACATTCGAAAGGCTGAAACAAATGCGTTTCGATGAATCGAGGTTAAAGACATATAATTCTCCTGAAGAGATGCGTGAACTTTTTC
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AGTGAACATTGGATATCAAACTGGTTCGTTTACATTCGAAAGGCTGAAACAAATGCGTTTCGATGAATCGAGGTTAAAGACATATAATTCTCCTGAAGAGATGCGTGAACTTTTTC
AGTGAACATTGGATATCAAACTGGTTCGTTTACATTCGAAAGGCTGAAACAAATGCGTTTCGATGAATCGAGGTTAAAGACATATAATTCTCCTGAAGAGATGCGTGAACTTTTTC
-------------------------------------------------------------------------------------------------------------------

**V**N**I**G**Y**Q**T**G**S**F**T**F**E**R**L**K**Q**M**R**F**D**E**S**R**L**K**T**Y**N**S**P**E**E**M**R**E**L**F**
**V**N**I**G**Y**Q**T**G**S**F**T**F**E**R**L**K**Q**M**R**F**D**E**S**R**L**K**T**Y**N**S**P**E**E**M**R**E**L**F**
**V**N**I**G**Y**Q**T**G**S**F**T**F**E**R**L**K**Q**M**R**F**D**E**S**R**L**K**T**Y**N**S**P**E**E**M**R**E**L**F**
**V**N**I**G**Y**Q**T**G**S**F**T**F**E**R**L**K**Q**M**R**F**D**E**S**R**L**K**T**Y**N**S**P**E**E**M**R**E**L**F**
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TTCACAAGAGCAGCAATGGCGGGATTGATGCTGCGTTCGATGAGGTCGCTTATATCAAGCTTTTCATGGCTAAGTATTGCTCAGAGTATTCCATCATCGAGCCTACCTTTAAGGCT
TTCACAAGAGCAGCAATGGCGGGATTGATGCTGCGTTCGATGAGGTCGCTTATATCAAGCTTTTCATGGCTAAGTATTGCTCAGAGTATTCCATCATCGAGCCTACCTTTAAGGCT
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TTCACAAGAGCAGCAATGGCGGGATTGATGCTGCGTTCGATGAGGTCGCTTATATCAAGCTTTTCATGGCTAAGTATTGCTCAGAGTATTCCATCATCGAGCCTACCTTTAAGGCT
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L**H**K**S**S**N**G**G**I**D**A**A**F**D**E**V**A**Y**I**K**L**F**M**A**K**Y**C**S**E**Y**S**I**I**E**P**T**F**K**A
L**H**K**S**S**N**G**G**I**D**A**A**F**D**E**V**A**Y**I**K**L**F**M**A**K**Y**C**S**E**Y**S**I**I**E**P**T**F**K**A
L**H**K**S**S**N**G**G**I**D**A**A**F**D**E**V**A**Y**I**K**L**F**M**A**K**Y**C**S**E**Y**S**I**I**E**P**T**F**K**A
L**H**K**S**S**N**G**G**I**D**A**A**F**D**E**V**A**Y**I**K**L**F**M**A**K**Y**C**S**E**Y**S**I**I**E**P**T**F**K**A
*******************************************************************************************************************

GATGGCTTTGGCTTTgtaagtggagctgattgcttttatttagacttttaatggtggcagcagaagagaggtacaccgcttgtagtcaaaaccacttttagctaacattcaaacg
GATGGCTTTGGCTTTGTAAGTGGAGCTGATTGCTTTTTATTTAGACTTTTAATGGTGGCAGCAGAAGA-----------------------------------------------
GATGGCTTTGGCTTTGTAAGTGGAGCTGATTGCTTTTTATTTAGACTTTTAATGGTGGCAGCAGAAGAGAGGTACACCGCTTGTAGTCAAAACCACTTTTAGCTAACATTCAAACG
GATGGCTTTGGCTTT----------------------------------------------------------------------------------------------------
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*D**G**F**G**F*****************************************************************************************************
*D**G**F**G**F**V**S**G**A**D**C**F**L**F**R**L**L**M**V**A**A**E**E***********************************************
*D**G**F**G**F**V**S**G**A**D**C**F**L**F**R**L**L**M**V**A**A**E**E**R**Y**T**A**C**S**Q**N**H**F**STOP***********
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*D**G**F**G**F*****************************************************************************************************
*******************************************************************************************************************

ttttggcctgtacgcaccaaacttgtgaacgatgaactaaaagcagctttctacactggcaccattcagaagtgatactaaatctcaatgattttgcagGCATTTCCACTAGGAT
---------------------------------------------------------------------------------------------------GCATTTCCACTAGGAT
TTTTGGCCTGTACGCACCAAACTTGTGAACGATGAACTAAAAGCAGCTTTCTACACTGGCACCATTCAGAAGTGATACTAAATCTCAATGATTTTGCAGGCATTTCCACTAGGAT
---------------------------------------------------------------------------------------------------GCATTTCCACTAGGAT
---------------------------------------------------------------------------------------------------GCATTTCCACTAGGAT
****************************************************************************************************A**F**P**L**G**
*****************************************************************************************************H**F**H**STOP
*******************************************************************************************************************
****************************************************************************************************A**F**P**L**G**
****************************************************************************************************A**F**P**L**G**

CTCCATTGGTGTCAGATATTTCAAGACAGATCTTGAACATAACAGAGGGAGATGCCATGAAAGCTATAGAGAACAAGTGGTTCCTTGGAGAAAAACATTGTCTGGACTCGACTACA
CTCCATTGGTGTCAGATATTTCAAGACAGATCTTGAACATAACAGAGGGAGATGCCATGAAAGCTATAGAGAACAAGTGGTTCCTTGGAGAAAAACATTGTCTGGACTCGACTACA
CTCCATTGGTGTCAGATATTTCAAGACAGATCTTGAACATAACAGAGGGAGATGCCATGAAAGCTATAGAGAACAAGTGGTTCCTTGGAGAAAAACATTGTCTGGACTCGACTACA
CTCCATTGGTGTCAGATATTTCAAGACAGATCTTGAACATAACAGAGGGAGATGCCATGAAAGCTATAGAGAACAAGTGGTTCCTTGGAGAAAAACATTGTCTGGACTCGACTACA
CTCCATTGGTGTCAGATATTTCAAGACAGATCTTGAACATAACAGAGGGAGATGCCATGAAAGCTATAGAGAACAAGTGGTTCCTTGGAGAAAAACATTGTCTGGACTCGACTACA
S**P**L**V**S**D**I**S**R**Q**I**L**N**I**T**E**G**D**A**M**K**A**I**E**N**K**W**F**L**G**E**K**H**C**L**D**S**T**T
*******************************************************************************************************************
*******************************************************************************************************************
S**P**L**V**S**D**I**S**R**Q**I**L**N**I**T**E**G**D**A**M**K**A**I**E**N**K**W**F**L**G**E**K**H**C**L**D**S**T**T
S**P**L**V**S**D**I**S**R**Q**I**L**N**I**T**E**G**D**A**M**K**A**I**E**N**K**W**F**L**G**E**K**H**C**L**D**S**T**T

TCAGATTCTCCAATCCAGCTCGACCACCACAGCTTTGAAGCTCTATTTCTGATCGTCTTTGTTGTTTCTGTGATTCTACTCTTACTCATGTTGGCTTCTAGAGGATACCAAGAGAG
TCAGATTCTCCAATCCAGCTCGACCACCACAGCTTTGAAGCTCTATTTCTGATCGTCTTTGTTGTTTCTGTGATTCTACTCTTACTCATGTTGGCTTCTAGAGGATACCAAGAGAG
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*S**D**S**P**I**Q**L**D**H**H**S**F**E**A**L**F**L**I**V**F**V**V**S**V**I**L**L**L**M**L**A**S**R**G**Y**Q**E**R
*******************************************************************************************************************
*******************************************************************************************************************
*S**D**S**P**I**Q**L**D**H**H**S**F**E**A**L**F**L**I**V**F**V**V**S**V**I**L**L**L**M**L**A**S**R**G**Y**Q**E**R
```

*S**D**S**P**I**Q**L**D**H**H**S**F**E**A**L**F**L**I**V**F**V**V**S**V**I**L**L**L**L**M**L**A**S**R**G**Y**Q**E**R

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ACAACACAATGCTTCACCCAATCTACCAAATGATCAAGCCAATGCAGCTCAAGAAGAAGTCAATGAAGAAGGTAATGTTGGAGATCATATTGTGGAAGTCGACACAGCTTTGGTCC
ACAACACAATGCTTCACCCAATCTACCAAATGATCAAGCCAATGCAGCTCAAGAAGAAGTCAATGAAGAAGGTAATGTTGGAGATCATATTGTGGAAGTCGACACAGCTTTGGTCC

QH**N**A**S**P**N**L**P**N**D**Q**A**N**A**A**Q**E**E**V**N**E**E**G**N**V**G**D**H**I**V**E**V**D**T**A**L*****

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QH**N**A**S**P**N**L**P**N**D**Q**A**N**A**A**Q**E**E**V**N**E**E**G**N**V**G**D**H**I**V**E**V**D**T**A**L**V**

QH**N**A**S**P**N**L**P**N**D**Q**A**N**A**A**Q**E**E**V**N**E**E**G**N**V**G**D**H**I**V**E**V**D**T**A**L**V**

gtcgtaagaaactgacctcaaacactatacccattagaagagttgcgccactatcaagGCTAAAGTCAGCATAGTCAAACCTAAACTCTAGatttctttcactgtttagattgcat
GTCGTAAGAAACTGACCTCAAACACTATACCCATTAGAAGAGTTGCGCCACTATCAAGGCTAAAGTCAGCATAGTCAAACCTAAACTCTAG
GTCGTAAGAAACTGACCTCAAACACTATACCCATTAGAAGAGTTGCGCCACTATCAAGGCTAAAGTCAGCATAGTCAAACCTAAACTCTAG
GTCGTAAGAAACTGACCTCAAACACTATACCCATTAGAAGAGTTGCGCCACTATCAAGGCTAAAGTCAGCATAGTCAAACCTAAACTCTAG
GTCGTAAGAAACTGACCTCAAACACTATACCCATTAGAAGAGTTGCGCCACTATCAAGGCTAAAGTCAGCATAGTCAAACCTAAACTCTAG

AK**V**S**I**V**K**P**K**L*

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R**R**K**K**L**T**S**N**T**I**P**I**R**R**V**A**P**L**S**R**L**K**S**A**STOP************

R**R**K**K**L**T**S**N**T**I**P**I**R**R**V**A**P**L**S**R**L**K**S**A**STOP************

Aatcataatatttaaggaatccttttctcaaatatgtttaaatgtatctaagatagactgatgcaaacttaaatcttacctactacta
5

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Jaar: **2014**

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De Blaes, Joris

Datum: **10/06/2014**