ABSTRACT

Thesis title: *ARABIDOPSIS THALIANA* GLUTAMATE RECEPTOR-LIKE 3.7 UNDERLIES ROOT MORPHOLOGY AND SIGNALING VIA MEMBRANE POTENTIAL HOMEOSTASIS

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Plants perceive highly variable environments and biotic interactions through membrane receptors like the GLutamate Receptor-like (GLR) family, related to the ionotropic Glutamate Receptors that underlie information transmission in neurons. GLRs underpin information transduction and morphological adaptations in plants. However, mechanistic understanding is scarce.

In *Arabidopsis thaliana* roots, we investigated how GLRs underlie amino acid-induced electric and $Ca^{2+}$ excitability. We also assessed the contribution of GLR3.7 in root hair elongation.

We present GLRs as mediators of a local, glutamate-induced electric and $Ca^{2+}$ response in roots, with the same initiation kinetics of wound-induced Slow Wave Potentials (SWP). We identify GLR3.7 as mediator of root hair elongation through maintenance of membrane depolarization at the growing cell apex.

These results propose a parallel between glutamate-triggered signals and SWP initial phase as local and chemically induced, and posit GLR3.7 as a possible contributor to $Ca^{2+}$ homeostasis in root hair apical growth.

# *ARABIDOPSIS THALIANA* GLUTAMATE RECEPTOR-LIKE 3.7 UNDERLIES ROOT HAIR ELONGATION AND ROOT ELECTRIC SIGNALING VIA LIGAND-DEPENDENT CALCIUM AND MEMBRANE POTENTIAL HOMEOSTASIS.

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# Dedication

*To*

*Alejandrina Santibáñez*

*y todos con los que volveré a compartir mesa.*

# Acknowledgments

I am thankful to all my lab mates and mentors for their patience, wise words, and support during this project.

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# Chapter 1: Introduction

Plants are capable of sensing their environment and accordingly respond with morphological and physiological adaptations. Perception of chemical signals is important for roots to sense the aqueous environment in the soil. But it is also essential for cell-to-cell communication through signaling molecules. Such signals can be detected by membrane receptors that induce an intracellular change in secondary messenger, like calcium ($Ca^{2+}$) (Feijó and Wudick, 2018). Plant genomes encode a family of GLutamate Receptor-like (GLR) channels, evolutionarily related to the ionotropic Glutamate Receptors (iGluR) in mammalian neurons, that seem to be essential in that step from chemical perception to intracellular $Ca^{2+}$ increase (Fromm and Lautner, 2007).

This research uses roots of the model plant *Arabidopsis thaliana* to achieve two aims: contribute to the dissection the GLR-mediated ligand-gated electric response. And, given the that root hairs are the only other type of apical growing cell in a plant besides pollen tubes (Michard et al., 2011), determine the role of GLRs in root hair growth.

## Glutamate Receptor-Like Channels

The genome of the model plant *Arabidopsis thaliana* encodes cDNA genes with the signature domains of the mammalian iGluR. These essential domains, tightly relate to the channel function; comprise three transmembrane domains and a Ligand Binding domain (LBD) (Green et al., 2021; Lam et al., 1998). This initial discovery was the first of a family of plant genes, the GLRs, that has a similar sequence to others found in different taxa along the evolutionary tree, form prokaryotes and primitive protist organisms to higher plants (Price et al., 2012, Wudick et al., 2018b). Particularly, the GLR family sequence seems to have an origin in the prokaryote potassium channels (Kuner et al., 2003). Individual species of algae encode one or two GLRs, but sequence variation is significant between taxa. Contrastingly, among seed plants the diversity is low but each taxon presents multiple homologue GLR genes. Their counts range from 9 in the evolutionarily primitive genus *Gingko*, to 38 in the higher plant genus *Brassica*; in between *Arabidopsis* is catalogued with 20 genes, divided in 3 clades (Bortoli et al., 2016).

While the close relation of GLR family to mammal iGluRs was deducted from sequence similarity analysis, a phylogenetic analysis, including bacterial glutamate receptors as reference outgroup, divided the family in three clades,

and determined that plant and animal iGluRs diverged from a common ancestor (Chiu et al., 1999; Price et al., 2012) (figure 1.1). The separation of

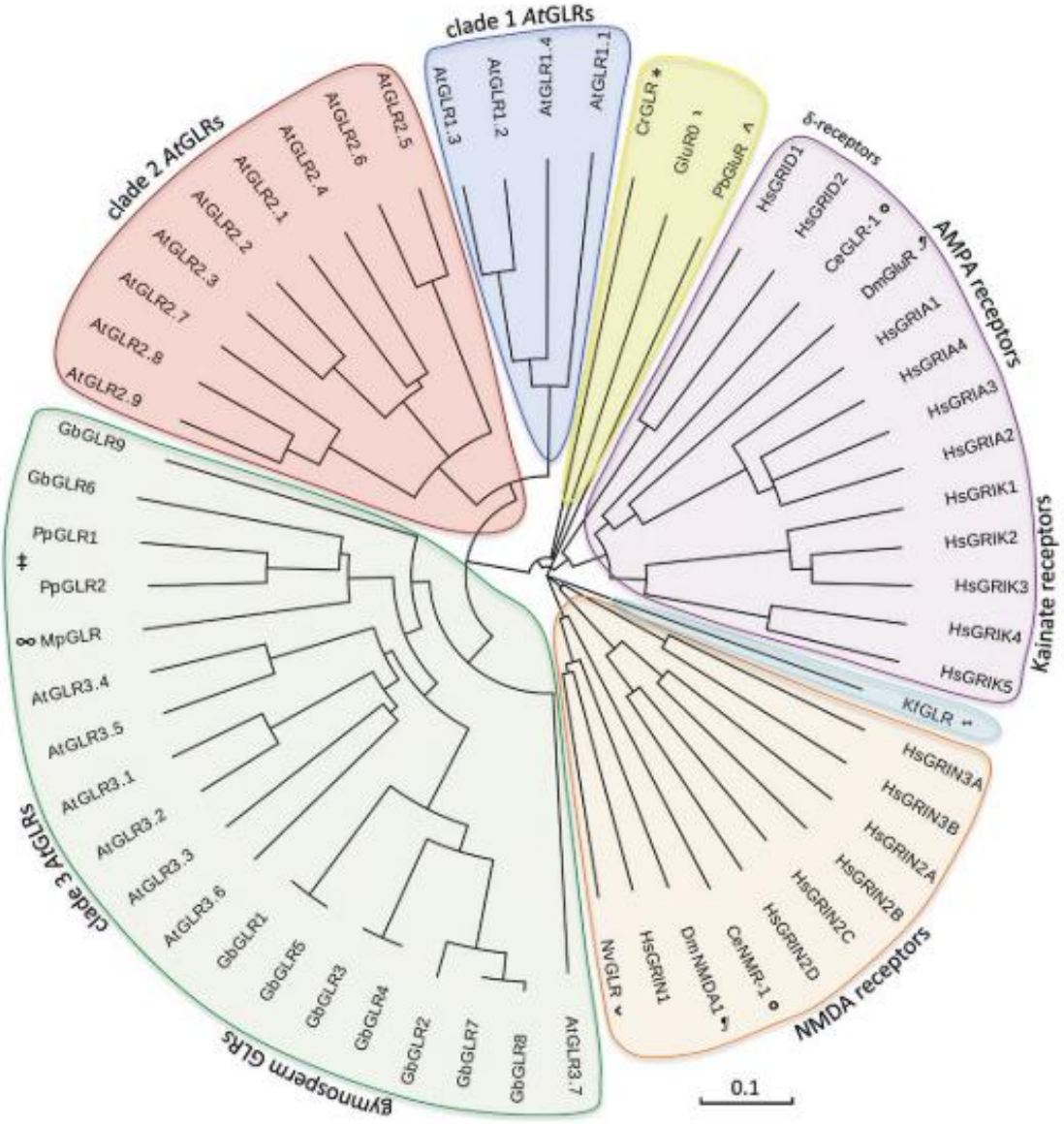



**Figure 1.1**. Phylogram of selected glutamate receptors from bacteria, plants, and animals. The tree shows the phylogenetic relationship of chosen glutamate receptors from Arabidopsis thaliana (At), the nematode Caenorhabditis elegans (Ce, °), the unicellular green alga Chlamydomonas rheinhardtii (Cr, *), the fruit fly Drosophila melanogaster (Dm, ¶), the gymnosperm Ginkgo biloba (Gb), Homo sapiens (Hs), the filamentous green alga Klebsormidium flaccidum (Kf, ¨), the liverwort Marchantia polymorpha (Mp, ∞), the sea anemone Nematostella vectensis (Nv, ˇ), the moss Physcomitrella patens (Pp, ‡), the ctenophore Pleurobrachia bachei (Pb, ^), and the cyanobacterium Synechocystis sp. (GluR0, ˜). The scale bar indicates substitutions per site (adapted from Wudick et al. 2018a).

these different genes seem to have happened very early in the evolution of cellular life, apparently, even as early as the separation of the Last Universal Common Ancestor (LUCA) (Price et al., 2012).

The GLR family phylogeny is well stablished. Among all the sequences, the putative, biophysically important segments of the channel, that determine ligand affinity and ionic selectivity, have short but potentially important variations. In *Arabidopsis*, clade 3 is the most ancient and more broadly expressed in *Arabidopsis*, while clades 1 and 2 are the most recent to evolve. Between the latter, the clade 2 is the most recent one and is the result of duplication events of clade 1. And although gene expression analysis suggests that all clades were expressed in the whole plant in *Arabidopsis*, now it is known that the clade 2 genes are restricted only to specific cell types, and presumably their functions in the rest of the plant are performed by other clades (Chiu et al., 2002; Price et al., 2012; Weiland et al., 2016).

The radiation of different clades, and the increase of numbers of copies in plant genomes occurred in parallel to land colonization (Bortoli et al., 2016), therefore it is thought that the variety of GLRs present today in vascular plants is a cornerstone of adaptation to dry land survival. Accordingly, GLRs have been related to stress related physiological traits such as stress hormones

Abscisic acid (ABA) biosynthesis and signaling (Kang et al., 2004; Kong et al., 2015), metabolic response to drought (Kang et al., 2004; Lu et al., 2014), innate immune response (Kang et al., 2004; Kwaaitaal et al., 2011; Li et al., 2013; Manzoor et al., 2013), stomatal closure (Cho et al., 2009), and wound-induced electric signaling (Mousavi et al., 2013) including response to aphid puncture into the phloem (Vincent et al., 2017). Also, the control of morphological characteristics and physiological processes native to land plants is related to GLR activity, e.g. primary root growth and secondary root initiation (Singh et al., 2016; Vincill et al., 2013), pollen tube apical growth (Michard et al., 2011; Wudick et al., 2018a), and flower-pollen self-incompatibility (Iwano et al., 2015). In moss, a early land plant, GLRs have been related to sperm chemotaxis, phase alternation in the life cycle (Ortiz-Ramírez et al., 2017), and tolerance to light deprivation (Simon et al., under review)

Focusing on *Arabidopsis* as the vascular plant model, the expression patterns of the three clades are different throughout the plant and its developmental stages. And the channels can function in different endomembranes besides the plasma membrane (Chiu et al., 2002; Lam et al., 1998; Teardo et al., 2011; Wudick et al. 2018a). Despite the multiplicity of channels and their

differential expression, the efforts to characterize their function have mainly focused on the clade 3, for its ubiquity and abundance (Hedrich et al., 2016; Kim et al., 2001).

## GLR Structure & Electrophysiology

In order to molecularly understand the mechanism by which GLRs are involved in the mentioned traits, it is fundamental to determine their channel characteristics. For this, elucidation of their electrophysiological and structural attributes has been a main focus.

GLRs similarity with ionotropic ligand-gated channels, postulates them as candidates for driving an electrochemical response to amino acids. This type of effect was early observed, but it was not clear if it was a by-product phenomenon of amino acid cotransporters or if it is a signal in itself (Dennison and Spalding, 2000; Etherton and Rubinstein, 1978). However, direct electrophysiological measurements of intracellular electric potential made evident a Glu-dependent depolarization, separated from amino acid-ion cotransporters, and mediated by GLRs (Demidchik et al., 2004; Qi et al., 2006).

Further characterization of GLR involvement in amino acid-dependent depolarization in roots proved that a variety of ligands can trigger a GLR-dependent response. Asparagine, glycine, cysteine, serine and alanine were screened along with glutamate (Stephens et al., 2008). All evoked depolarization in root cells, being glutamate the one with a lowest half maximum concentration but not the highest maximum response (Stephens et al., 2008). This low specificity of GLR3.3 to amino acids, and the variety of responses depending on the ligand, is not the same as in mammalian iGluRs, which have a higher ligand specificity (Hollmann et al., 1989; Traynelis et al., 2010).

Similar to iGluRs, GLRs can form homo- or heterotetramers as their functional quaternary structure in the membrane (Price and Okumoto, 2013; Green et al., 2021; Simon et al., under review). If the modulation of ligand affinity by heterotetrameric composition observed in iGluRs is effective in GLRs, and given their variable ligand specificity, then the different possible combinations of GLR tetramers and ligand affinity will result in an broader sensitivity than that of iGluRs (Alfieri et al., 2020; Yao et al., 2008).

To understand how GLRs can bind several amino acids, and then, how this can change in heterotetramers, it is important to dilucidate the physical interactions for the ligands with the protein structure. The general structure of eukaryotic glutamate receptors consists of tetrameric conformation in a "Y" shaped transmembrane protein. It has three distinctive layers: an amino-terminal domain (ATD), a ligand-binding domain (LBD), and a transmembrane domain (TMD) (figure 1.2).

The full-length structure of the *Arabidopsis* GLR3.4 is the first one dilucidated in the family. It has elements of all subtypes of iGluR, and provides valuable insights of the gating and permeation mechanism (Sobolevsky et al., 2009; Green et al., 2021).

The ATD fractions also allow interaction between the monomers, aid channel assembling and provide tetrameric stability. The structure of the ATD resembles a clamshell that can open or close when interacting with a ligand. In the AMPA and kainate type receptors it has not been found to functionally bind to any ligand, maintaining a closed clamshell structure (Jin et al., 2009). The ATD of the GLR3.4 seems to be capable of binding peptides, however the data is yet not conclusive (figure 1.2-A) (Green et al., 2021), a ligand that

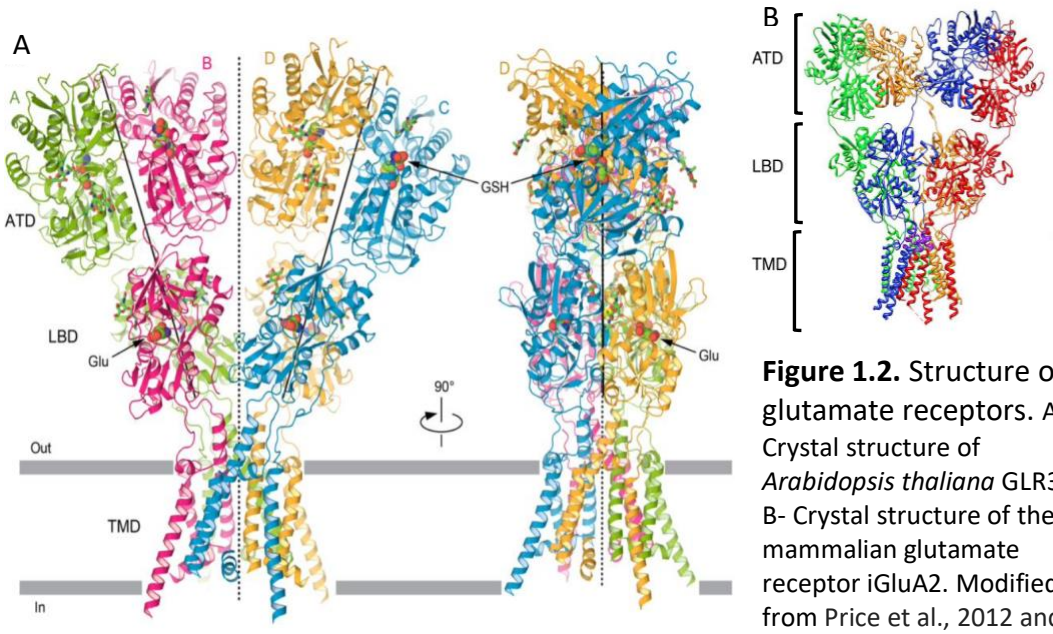



**Figure 1.2.** Structure of glutamate receptors. A- Crystal structure of *Arabidopsis thaliana* GLR3.4. B- Crystal structure of the mammalian glutamate receptor iGluA2. Modified from Price et al., 2012 and Green et al., 2021).

mediates GLR-dependent electric excitability (Qi et al., 2006), and activation of immune response genes (Li et al., 2013). In GLR3.4 the ATD is connected to the layer beneath, the LBD, through a network of electrostatic and hydrophobic interaction that transfer conformational changes. Such changes can be induced by ligand binding in the ATD (Green et al., 2021).

Another form of channel structural regulation relies on the LBD, which is closer to the TMD that conforms the permeating pore. In iGluRs the LBD typically mediates the conformational changes that open the channel. In the case of *Arabidopsis* GLRs, the LBD of GLR3.4, GLR3.3, and GLR3.2 have been isolated and determined to have a clamshell architecture structurally

similar to that of iGluRs, that structurally change upon ligand binding (Alfieri et al., 2020; Gangwar et al., 2020). It was also observed that the LBD pocket of GLRs can bind to a variety of amino acids, unlike that of iGluRs, by having certain conserved binding sites and, when needed, filling the space of the amino acid side chain with water molecules or sodium ($Na^+$) ions (Gangwar et al., 2020; Green et al., 2021).

The TMD section conforms the ion channel that determines the selectivity and the conductance. The GLR3.4 channel is formed by a set of residues that differ from that of iGluRs, indicating different permeability properties (Green et al., under review). This was confirmed using a patch clamp experimental setup that demonstrated permeability of anions, as well as cations, conferred by heterologous expression of the moss *Physcomitrium patens* GLR1, and *Arabidopsis* GLR3.4 and GLR3.2 (Simon et al., under review). From this type of experiments it was also determined that moss GLR1 and a set of clade 3 *Arabidopsis* GLRs have a basal open conductance even in the absence of a ligand (Ortiz-Ramírez et al., 2017; Wudick et al., 2018b).

Present functional and electrophysiological evidence converge to demonstrate that the mechanism of ligand binding is conserved, but the channels

permeability is drastically divergent in mammalian iGluRs and plant GLRs (Alfieri et al., 2020; Gangwar et al., 2020; Simon et al,. under review; Green et al., 2021). This raises the question, why did GLRs evolve a different permeability? The derived trait could be an adaptation for maintaining a depolarizing role in cells where $Na^+$ influx is toxic (Wudick et al., 2018b), but the ample variability of GLRs and their tetrameric combinations could configure their ligand and permeation specificities for many other functions.

For this reason, and despite the novel partial anionic selectivity presented above, GLRs have traditionally been identified as $Ca^{2+}$ permeable channels. For example, the application of amino acids to root cells might initiate a GLR3.3 dependent depolarization that could be abolished by $Ca^{2+}$ channels specific antagonists (Davenport, 2002; Dennison and Spalding, 2000; Qi et al., 2006). Like this, there is broad evidence that links GLRs with electric signaling, a conserved manner of interacting with the environment, transverse to multiple evolutionary distant clades, and coordinated by ionic channels (Volkov, 2006). Thus, electric signaling in plants is essential for the study of GLR function.

## Origins of plant electrophysiology

To understand the pivotal differences between plant and animal electrophysiology, why the latter is more developed, and how the particularities of bioelectricity in plants has been a barrier for understanding the underlying channel function, it is necessary to see a parallel of the electrophysiology's milestones in both natural kingdoms.

The concept of electricity as information carrier in biological systems was prominently documented in 1791 when the Italian physicist Luigi Galvani prepared an isolated frog leg that contracted upon contact with different types of metals (Galvani, 1791 cited from (Piccolino, 1997)). These observations, not only triggered the field for the development of electrical batteries, but also led to the creation of electrophysiology as a study field (Piccolino, 1997; Stahlberg, 2006 in (Volkov, 2006)).

Galvani performed public experiments and published his observations, nonetheless the governance of electricity over living tissues was accepted by the European scientific community only in 1844, when Carlo Matteucci took over and clearly demonstrated that living tissues can generate electricity autonomously. The Italian physicist generated a circuit of frog legs connected

in series, showing that their currents can be summed as if they were batteries (Verkhratsky and Parpura, 2014 in (Martina and Taverna, 2014)).

Once bioelectricity settled in the scientific community, proper description of the phenomenon, and its observation in diverse living forms flourished. The first electric transients, now called Action Potentials, were recorded in carnivorous plants leaves and frog leg muscles using extracellular (contact) electrodes.

The first extracellular recording of a muscular action potential (AP) was recorded by Emil du Bois-Reymond, a signal he called "a negative fluctuation". Shortly after, and promoted by Darwin's idea that the carnivorous plant *Dionea muscipula* presents animal-like reflexes, extracellular electric potential waves in the closing leaves were recorded using the galvanometer developed by Galvani and Matteucci (Burdon and Sanderson, M, 1873; Darwin, 1875; Verkhratsky and Parpura, 2014 in (Martina and Taverna, 2014)).

By the same time, in 1871, the first intracellular recording was attempted in severed nerves by Julius Bernstein (Nilius, 2003). But this type of recording became accessible only with the implementation of glass pipettes as electrodes that could measure the intracellular potential with minimum harm to the cell.

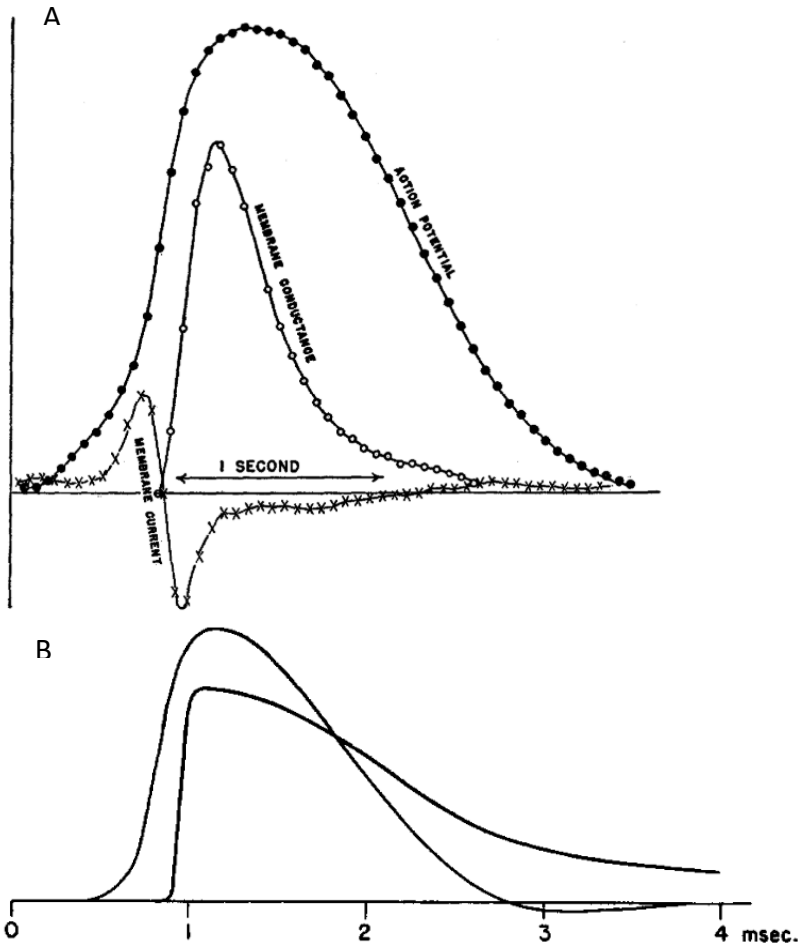



**Figure 1.3**. Traces of intracellular recording of AP in (A) the algae *Nitella*, and (B) *Loligo* squid giant axon, the trace with a bigger amplitude represents the membrane potential and the other represents the membrane conductance. Adapted from (Cole and Curtis, 1938, 1939)

Intracellular membrane potential recordings were first accomplished in the big internodal cells of algae of the genus *Nitella*, showing a resting membrane

potential around -100mV and transient electric responses upon electric stimulation (Umrath, 1930). Shortly after, Hodgking and Huxley accomplished the recording of an AP in the nerve of the squid *Loligo forbesi*, and corroborated Bernstein's original observation of an animal cell resting membrane potential around -60 mV (Hodgkin and Huxley, 1939). Then, Cole and Curtis showed the mechanistic similarities between AP in axons and algae cells (figure 1.3) (Cole and Curtis, 1938, 1939)

From this point the study of electrophysiology in plant cells diverged from animal cells, arguably because, while specialized cells for electric transmission, nerves, were identified in animals (Young, 1936), plants lack cells with this characteristic and electric signals propagate mainly in the vascular tissue, which is hardly as accessible as a squid axon (Bose, 1926). Also, the interconnectivity of plant cell through plasmodesmata, electrically considered a low resistance leak, demands a different, more complex approach for clamping the voltage of a single cell.

Comprehension of electric transmission in plants has proven to be more complex than the inaccessibility of the vascular tissue. Electric signals can take different shapes, fast depolarizations and repolarizations or long-lasting

depolarizations. And their propagation mechanism can be attributable to a synergy of various physical phenomena: electric propagation, molecular diffusion, and water pressure waves (Farmer et al., 2020; Hedrich et al., 2016).

## Electric signaling, GLRs, and physiological functions

Despite the intricated and not fully understood propagation mechanism of electric signals, their generation has been linked to a multiplicity of stimuli, and their effect to a series of physiological responses. Leaf cells depolarize upon light exposure, and can elicit APs when crossing a threshold (Fromm and Lautner, 2007; Kreslavski et al., 2009); in *Arabidopsis*, light induced electric signals can travel along the plant and alter photosynthetic metabolism (Szechyńska-Hebda et al., 2010, 2017); rehydration of the dry soil that hosts grape and avocado plants evokes propagating depolarizations throughout the root and shoot (Gil et al., 2008, 2014) and intake of amino acids into cells causes depolarization (Etherton and Rubinstein, 1978); also pressure change in the root system of pea plants can initiate a propagating electric signals (Stahlberg and Cosgrove, 1997); the style of lily flowers generates a propagating electric signal upon contact of pollen grain, and regulates compatibility to avoid self-crossing (Spanjers, A, 1977; Spanjers, 1981).

Lastly, tissue wounding elicits a propagating depolarization that is widespread in the plant kingdom (Farmer et al., 2020; Fromm and Lautner, 2007; Rhodes et al., 1999). This type of electric signal is well understood and progress has been done at identifying the channels implicated. Of particular interest, it is initiated by GLRs of the clade 3, and generates a defense response in far-away tissues, which activates genes associated with the jasmonates and ethylene pathways, use by the plant as a response to stress (Acosta and Farmer, 2010; Marhavý et al., 2019; Mousavi et al., 2013; Nguyen et al., 2018; Tran et al., 2018).

The type of electric signal initiated by tissue wounding is called Slow Wave Potential (SWP) (Fromm and Lautner, 2007), and has initially reported in *Mimosa* (Houwink, 1935), and then also identified in bean, tomato (Roblin, 1985), sunflowers, cucumbers (Stahlberg and Cosgrove, 1996, 1997; Stanković et al., 1997), wheat (Vodeneev et al., 2012) and *Arabidopsis* (Mousavi et al., 2013).

*Arabidopsis* plants lacking expression of either *GLR3.1, 3.2, 3.3* or *3.6* have an attenuated SWP, and in the double mutant *glr3.3-6* there is no electric signal initiated by wounding, nor the cytosolic $Ca^{2+}$ increase associated with

it (Mousavi et al., 2013; Nguyen et al., 2018). Besides this, and in accordance with structural data previously presented, stimulation of roots with Glu, GSH, and other amino acids elicits membrane depolarization and $Ca^{2+}$ increase. A response that is diminished in *glr3.3* plants (Alfieri et al., 2020; Demidchik et al., 2004; Qi et al., 2006). It is possible that GLRs mediate a SWP in plant tissues but do not comprise all the conductances implicated in the depolarization phase (Farmer et al., 2020). Disentangling the precise role of GLRs in SWPs is a challenging task because of the multicellular nature of the signal, and the technically difficult task of clamping the voltage of an interconnected network of cells for current isolation. Thus, the relatively simpler, cable-like geometry of roots, along with other reasons presented next, posit them as an advantageous organ on which to understand the role of GLRs in signaling.

## GLR functions in roots

Root cells protoplast are electrically excitable, permeate $Ca^{2+}$ and $Na^{+}$ triggered by Glu in a dose-dependent manner. These current convey a transient depolarization that can be abolished by the application of non-selective cation channel blocker, like $La^{3+}$ and $Gd^{3+}$, these currents were

indirectly attributed to GLR3.3 via a reverse genetics approach (Demidchik et al., 2004; Dennison and Spalding, 2000; Qi et al., 2006).

The implication of GLRs in wound response and $Ca^{2+}$ signaling spans to other plant species. In tomato roots a long-distance electric signal has also been reported in response to nematode infestation. This signal, abolished in *Slglr3.5* plants, travels in parallel with a wave of increasing Reactive Oxidative Species (ROS), and lastly initiates the jasmonate pathway as a defense response (Wang et al., 2019). The initiation of electric and $Ca^{2+}$ transients is, however, not the only effect Glu has on roots, nor the only function of GLRs. In *Arabidopsis*, roots are the only organ that express all the 20 GLRs of the genome (figure 1.4) (Chiu et al., 2002; Turano et al., 2002), which suggests their implication in multiple signaling pathways and physiological responses.

Root elongation occurs by mitotic activity of the meristem at the root tip. The regulation of this process provides the root the ability of directing the growth and regulating the elongation pace. Application of exogenous Glu decreases the elongation speed of roots by decreasing the mitotic activity at the primary root tip (Skobeleva et al., 2011; Walch-Liu et al., 2006). GLR3.6 is strongly localized at the root tip, particularly in the cells that comprise the apical

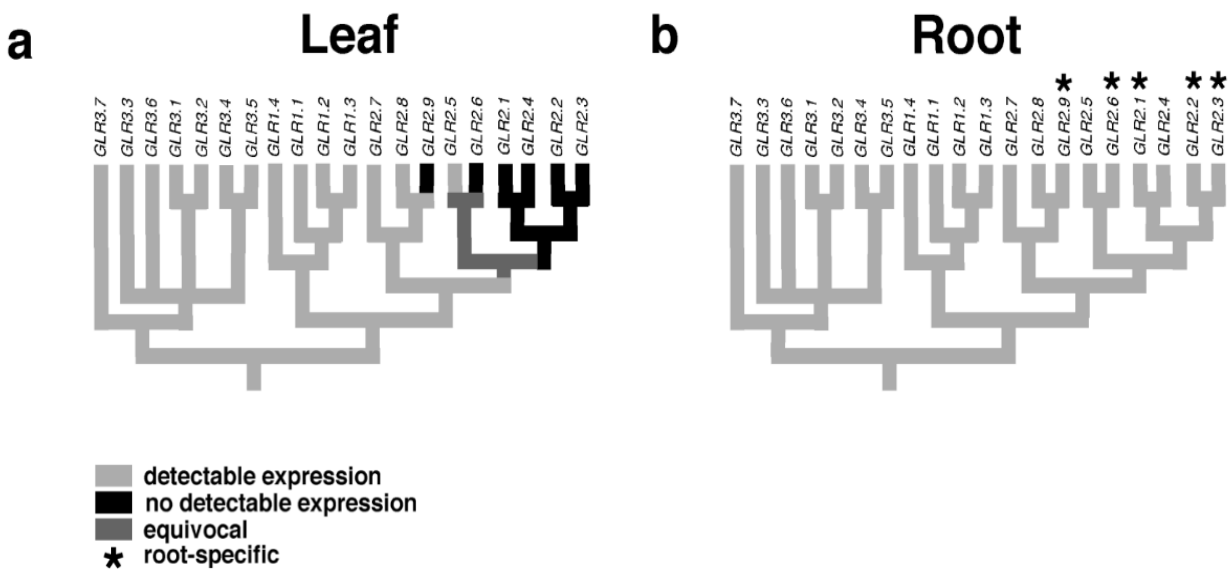



**Figure 1.4.** Expression pattern of the *Arabidopsis thaliana* GLR members in (a) leaves and (b) roots.
Modified from Chiu et al., 2002

meristem and, at the mature zone of the root, in the vascular bundle only. Interestingly, in *glr3.6* mutant plants the growth rate is decreased in comparison to the wild type. This was related to upregulation of a cell cycling inhibitor, decreased mitotic rate and apical meristem size, and diminished endogenous auxin production (Singh et al., 2016).

The apparent contradiction of exogenous Glu application and GLR3.6 knockout having the same effect demonstrates the complexity of cell signaling related to GLRs, and requires further investigation.

Additionally, as the root tip grows the mature region starts generating primordia from which lateral roots are formed. The signal that initiates the

differentiation of primordia is related mainly to auxin, but exogenous Glu application can also regulate it, by increasing the density of lateral roots (Walch-Liu et al., 2006), and decreased in the *glr3.6* mutant (Singh et al., 2016). In contrast, *glr3.2* and *glr3.4* mutants have an increased lateral root density (Vincill et al., 2013). While *glr3.3* presented no change in the amount of lateral root primordia, it did display a more unstable gravitropic response (Miller et al., 2010; Vincill et al., 2013)

Roots present a specialized cell type, called trichoblasts, that differentiate from epidermal cells and form root hairs (reviewed in (Grierson et al., 2014)). The differentiation of trichoblasts is regulated by ethylene, a gaseous molecule metabolized from the non-proteogenic amino acid 1-aminocyclopropane 1-carboxilic acid (ACC) (Tanimoto et al., 1995). The elongation of trichoblasts to form root hairs is tightly regulated by $Ca^{2+}$ increase at the growing tip (Wymer et al., 1997). We hypothesize that GLRs are implicated in this process since GLR1.2 and GLR3.7 are related with tip elongation of pollen tubes in *Arabidopsis* (Mendrinna and Persson, 2015; Michard et al., 2011).

## Overlap between GLR signaling and root hair development

Root hairs form from trichoblasts, specialized epidermal cells that differentiate in a tightly controlled pattern. Only the epidermal cells that are in contact with two cells in the inner layer become trichoblasts, but application of exogenous ACC induces differentiation of cells that do not comply with this condition (Dolan, 2001; Tanimoto et al., 1995). The induction of differentiation and elongation by ethylene is mediated by the ethylene pathway member protein Ethylene Insensitive 2 (EIN2) (Martín-Rejano et al., 2011).

Apical growth is a specific process of cell development in which elongation occurs only at the cell apex, and is highly conserved in pollen tubes and root hairs (Campanoni and Blatt, 2007; Candeo et al., 2017; Feijó et al., 2004). Both cell types develop an apical $Ca^{2+}$ increase that drives elongation (Schiefelbein et al., 1992; Vissenberg et al., 2001; Wymer et al., 1997). In *glr1.2* and *glr3.7* the homeostasis of $Ca^{2+}$ at the growing tip is altered, thus elongation rate is diminished (Michard et al., 2011).

Furthermore, apical elongation of root hairs happens in an oscillatory pattern that is tightly correlated to intracellular $Ca^{2+}$ oscillations that stops when the

cell reaches its final length (figure 1.5) (Candeo et al., 2017; Wymer et al., 1997). Although it is hypothesized by Mendrinna and Persson (2015), there is no evidence that GLRs are implicated in the $Ca^{2+}$ homeostasis in elongating root hairs.

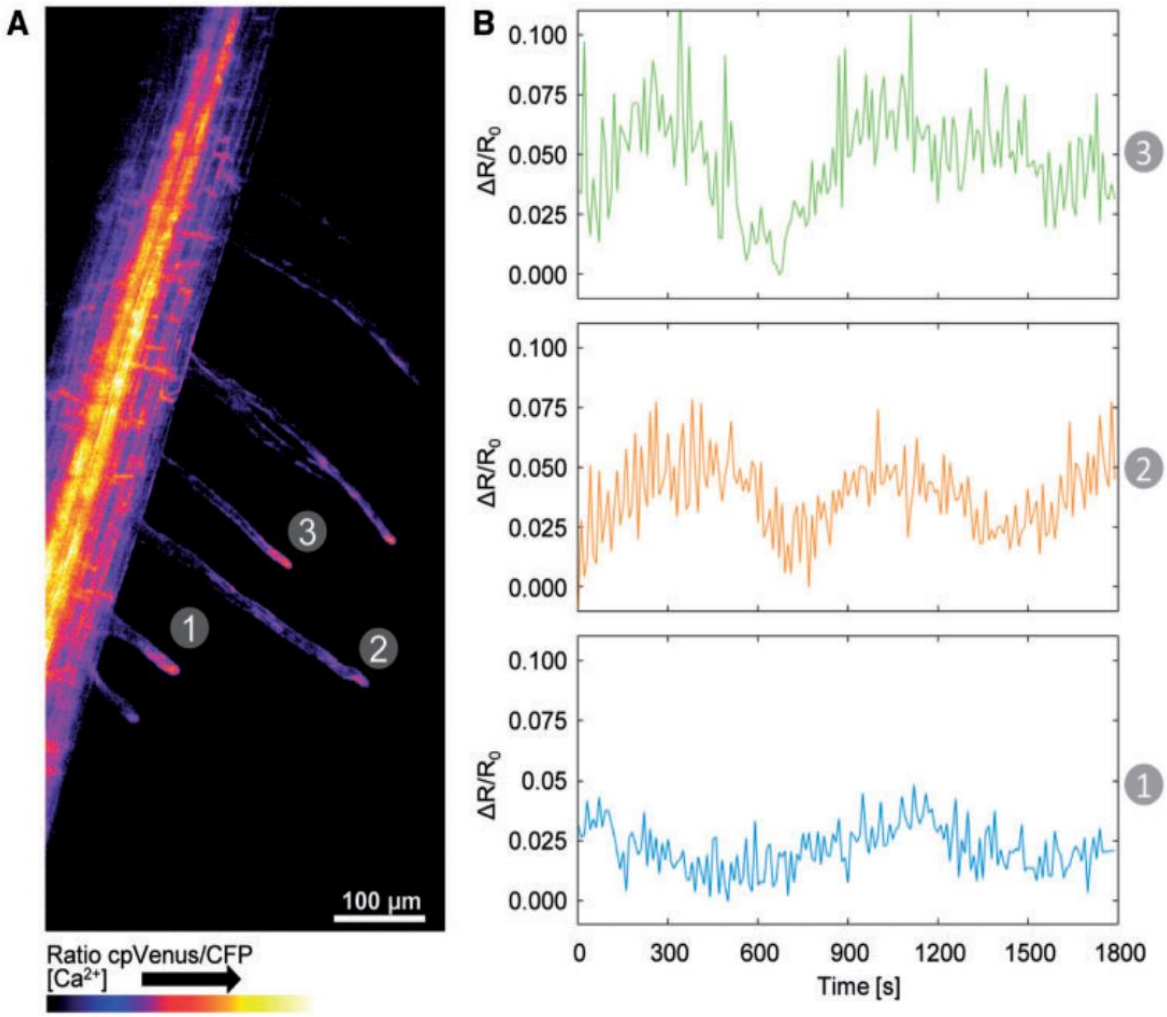



**Figure 1.5.** Intracellular $Ca^{2+}$ imaging in *Arabidopsis* root hairs expressing the fluorescent reporter YC3.6. A – Root hairs present higher Ca2+ concentration at the tip, and disappears when the cell stops growing. B – oscillatory pattern of cytosolic Ca2+ during root hair elongation. Modified from Candeo et al., 2017

There is available evidence that shows the multiple roles of $Ca^{2+}$ in apical elongation, and give base to the hypothesis that GLRs have regulate $Ca^{2+}$ homeostasis at the tip of growing root hairs. The ethylene precursor ACC activates depolarization and $Ca^{2+}$ influx in protoplasted root epidermal cells. This occurs by activation of various GLRs, mainly GLR3.6 and GLR3.3, and in a decreased magnitude GLR3.1 and GLR3.2 (Mou et al., 2020). Complementary, single cell wounding in roots induces $Ca^{2+}$ increase and ethylene production (Marhavý et al., 2019). Moreover, GLRs mediate $Ca^{2+}$ and electric transients in roots in response to amino acids (Qi et al., 2006) and wounding (Shao et al., 2020).

Additionally, intracellular $Ca^{2+}$ concentration and membrane depolarization are also correlated with cytoskeleton stability during cell expansion. In the case of the moss *Physcomitrium patens*, apical growth also presents $Ca^{2+}$ oscillations that correlate to growth. Actin filaments disassembly is induced during high $Ca^{2+}$ concentration, and assembly during decreased concentration (Bascom et al., 2018; Bezanilla and Perroud, 2018). In root hairs, a turnover of actin filaments at the cell tip has also been reported (Ketelaar, 2013). Also microtubules are essential for sustaining proper apical growth (Bibikova et al.,

1999), and their integrity can be altered by exogenous Glu application through depolarization and $Ca^{2+}$ increase (Sivaguru et al., 2003).

Altogether, this presents the possibility of a feedback between $Ca^{2+}$, ethylene and amino acids, that is based on GLR activity, and underlies the signal that promotes the subcellular processes that compose root hair elongation and electric response. This research aims to determine the function of GLRs in electric and $Ca^{2+}$ signals that are transverse to wounding response and root hair elongation. We hypothesize that the electric and $Ca^{2+}$ responses of root cells to Glu are not self-propagating, and might correspond to part of the signal triggered by wounding. Also, root hair apical growth is mediated by strongly gated GLRs like GLR3.7.

# Chapter 2: GLRs mediate Glutamate-triggered local electric and $Ca^{2+}$ transients resembling initial phase of SWP.

## Introduction

### *Electric signals in plants*

Electric signals in biological systems occur as transitory changes in the cell membrane potential. This is mediated by a flow of ions that carry an electric current. Ionic flow can be mediated by (1) a group of functionally interconnected membrane channels that drive depolarization and repolarization or (2) leakage product of membrane perturbation, like in the cases of wounding. In many cases electric signals culminate in a free cytosolic calcium ($Ca^{2+}$) concentration increase that conveys other signaling input for the rest of the system. Information transmission in animal nerves is encoded in such electric signals, which can spatially self-propagate due to the electrical excitability of nerve membranes (Kandel et al., 2013).

Plant cells also convey information by alteration of the membrane potential (reviewed by Fromm and Lautner, 2007). Electric signal in plants have been reported to coordinate mechanical movements in the carnivorous plant

*Dionaea muscipula* (Burdon and Sanderson, 1873) and the legume *Mimosa pudica* (Bose, M, 1913; Houwink, 1935). Curiously, the filamentous *Nitella* was used to record the first action potentials ever using intracellular microelectrodes (Umrath, 1930). Electric signals were described in pistils upon pollination of corn and rapeseed (Mól et al., 2004; Wędzony and Filek, 1998), where they mediate self-incompatibility and fruit formation as described in Lily (Linskens and Spanjers, 1973; Spanjers, 1981). Electric signals were also observed in responses to wounding (Wildon et al., 1992) and have role in the elicitation of the innate immune response through the jasmonate pathway defense mechanism (Acosta and Farmer, 2010; Mousavi et al., 2013; Nguyen et al., 2018; Szechyńska-Hebda et al., 2017).

Unlike the electric signal of constant velocity and amplitude in the animal nervous system (Hille, 2001), electric signals in plants have diverse kinetics and propagation mechanisms, and have been proposed to be compartmentalized, by virtue of differential mechanistic causes, in system potentials (SP), action potentials (AP), and slow wave potentials (SWP; also known as variation potentials) (Farmer et al., 2020).

System potential have been described as slow (5-10 $cm.min^{-1}$) hyperpolarizing signals evoked by application of cations to injured leaves (Zimmermann et al., 2009). AP in plants can propagate autonomously at a speed of 2-3 $cm.s^{-1}$ (Fromm and Lautner, 2007), and are considered analogous to AP in animal nerves. But unlike using sodium ($Na^+$; influx for de-polarization) and potassium ($K^+$; for re-polarization) to form APs in animals, plant APs derives from fluxes of chloride ($Cl^-$; efflux for de-polarization), $Ca^{2+}$ and $K^+$, which are mediated by changes in the conductance of excitable membranes by a repertoire of channels not totally understood in plants (Cuin et al., 2018; Felle and Zimmermann, 2007).

SWP are long-lasting, transient depolarizations that rise in a couple seconds and have a prolonged repolarization that can last up to minutes. This type of signal propagates from injuries like wounding or burning at a speed in the range of $cm.min^{-1}$ (Stahlberg and Cosgrove, 1996; Stahlberg et al., 2006). The propagation is electrotonic i.e., it does not rely on electrically active membranes, as occurs in APs. Hence, the propagation mechanism of SWPs is still a matter of debate. One theory states that the signal travels as a change of hydraulic pressure transmitted through the xylem upon cellular damage (Farmer et al., 2014; Stahlberg and Cosgrove, 1997). In contrast, it is proposed

that SWPs can be initiated by chemical cues released by damaged cells and diffuse quickly in the turbulent flow of the xylem (Sukhov et al., 2013; Vodeneev et al., 2012).

### *Molecular mechanisms underlying electric signals in plants*

Electric excitability among different plant clades is driven by depolarizing anionic ($Cl^-$, malate, nitrate) currents and repolarizing $K^+$ currents (Cuin et al., 2018; Felle and Zimmermann, 2007; Homann and Thiel, 1994). APs in the more primitive algae model *Chara* have been thoroughly studied, and the participating channels have been identified (Beilby, 2007). However, the molecular identity and coupling mechanism of the different channels that underpin electric signals is not totally clear in higher plants.

Charales is a group of fresh water green plants. It is the living aquatic plant clade most closely related to land plants (Karol et al., 2001). Specifically, plants in the genus *Chara* have branching nodes interconnected by long cells. The size of these internodal cells allowed early intracellular electrophysiological recordings (Umrath, 1930).

Electric stimulation of internodal cells can readily evoke an AP with an average duration of 1 s and an amplitude of around 60 mV (Homann and Thiel, 1994). The conductances implicated in this transient depolarization and slow repolarization have been identified by single channel recordings and ionic concentration manipulation. The depolarization is initiated by increase of cytoplasmic $Ca^{2+}$ that comes from intracellular stores (Plieth et al., 1998). Then, depolarization is driven by $Cl^-$ efflux through voltage-dependent (Coleman, 1986) and, based on electrophysiological characterization, at least two types of voltage-independent channels are activated by $Ca^{2+}$ (Beilby and Al Khazaaly, 2016; Homann and Thiel, 1994). The molecular identification of these channels is still contentious. Finally, depolarization-activated $K^+$ channels mediate an outward rectifying conductance that repolarizes the membrane potential, and provokes a transient hyperpolarization (Beilby, 2007; Homann and Thiel, 1994).

In land plants, channels that mediate fluxes of $K^+$, $Cl^-$, and $Ca^{2+}$ have experimentally been proposed as mediators of depolarizing electric signals, a hypothesis that received support by computer simulations (Sukhov et al., 2011, 2013). Specifically, APs and SWPs are underpinned by a series of feedback loops similar to the ones described in algae; first, in SWPs, cytosolic

$Ca^{2+}$ induces fast anionic depolarization and posteriorly $K^+$ mediated repolarization, while a long term $H^+$ ATPase inhibition maintains the observed prolonged depolarization (Felle and Zimmermann, 2007; Sukhov et al., 2013). In contrast, APs in land plants present the same $Ca^{2+}$ initiation, anionic depolarization and $K^+$ repolarization, but there is no long-lasting inhibition of the $H^+$ pump (Sukhov et al., 2011; Vodeneev et al., 2006), hence the repolarization of APs is considerably faster than that of SWPs.

### *Genetic identity of electric signal mediators*

The molecular identity of the anionic channels involved in electric signaling is not known but several candidates have been proposed, namely those that mediate bulk ionic fluxes and drive depolarization and stomatal opening through water movement (Roelfsema et al., 2012). Those channels were initially classified by electrophysiology, as defining two independent anion based de-polarization profiles, one slow and one fast. Molecular characterization later showed these activities to be grossly attributable to members of the families of the Slow Activating Anion Channel (SLAC) and the Aluminum activated Malate Transporter channel (ALMT), respectively, identified in guard cells of *Vicia Faba* (Dietrich and Hedrich, 1998; Raschke

et al., 2003), *Nicotiana tabacum* (Stange et al., 2010), and *Arabidopsis* (Imes et al., 2013; Meyer et al., 2010; Mumm et al., 2013).

In particular, ALMT channels, which in Arabidopsis and other species do not transport Aluminum, were originally classified as rapid (R-type) anion channels, due to their voltage-dependent gating response in the range of 100 ms (Meyer et al., 2010; Mumm et al., 2013), and have been hypothesized to be implicated in the rapid membrane potential depolarization of AP and SWP. This is supported by theoretical models but not yet experimentally shown (Hedrich et al., 2016; Sukhov et al., 2013).

Other type of anionic channels likely implicated in electric signaling are the members of the stretch activated channel family MSL. This family of 10 channels is homologue to the bacterial Mechanosensitive channels of small Conductance (MscS), and despite the low sequence conservation, these channels provide effective mechano-sensitivity to plant cells (Monshausen and Haswell, 2013). The family member MSL10 has been shown in *vitro* to permeate anions upon mechanical stimuli (Maksaev and Haswell, 2012), to mediate mechanically activated depolarization in *Arabidopsis* root cells (Haswell et al., 2008), and its homologue in the carnivorous plant *Dioneae*

*muscipula* has been found highly expressed in electrically excitable cells (Procko et al., 2020). It is hypothesized that MSL10 can initiate electric signals in *Arabidopsis* (Guerringue et al., 2018).

In contrast, some $K^+$ and $Ca^{2+}$ channels have been genetically identified as active mediator of electric signals. Outward rectifying $K^+$ channels were early reported in plant membrane excitability (Blatt and Thiel, 1994). There are several genes in *Arabidopsis* that encode this type of channels: the Shaker family has several members that have been described as outward rectifying and voltage dependent (Véry and Sentenac, 2002). The Guard cell Outward Rectifying $K^+$ channel (GORK), is a member of the Shaker superfamily (Ache et al., 2000). It is a depolarization-activated, outward rectifying $K^+$ channel that limits the amplitude of the anionic depolarization and partially mediates the repolarization (Cuin et al., 2018; Riedelsberger et al., 2015).

Regarding the Ca2+ conductance active during electric signals, the Glutamate Receptors-Like channels (GLR) have been proposed as responsible for the cytoplasmic $Ca^{2+}$ increase that precedes depolarization. *Arabidopsis* plants with no expression of GLR3.3 and GLR3.6 present a small depolarization on the wounding site but no SWP propagation in response to that stimulus (Mousavi et al., 2013; Nguyen et al., 2018).

*Propagation mechanisms of electric signals*

The channels implicated in electric excitability have been identified in AP and SWP and used interchangeably as base to propose mechanistic theories of both types of electric signals. The use of channels described in one type (AP or SWP) to drive conclusions in other type of electric signals can be troublesome due to their inherent different propagation mechanisms. E.g. the implication of GLRs as depolarizing, $Ca^{2+}$ permeable channels, was described during wound-induced SWP (Nguyen et al., 2018), and the role of GORK in membrane potential repolarization was described in an electrically induced AP (Cuin et al., 2018). In particular, GLRs as mediators of $Ca^{2+}$ initiated depolarization in SWP is hard to apply to AP initiation due to the non-electric SWP propagation mechanism, and the yet unknown voltage-sensitivity of GLRs.

Moreover, AP is an electric signal that actively propagates while SWP is a local response to a propagating signal that can be mechanical or chemical (Sukhova et al., 2017). SWP propagation relies on a ligand or mechanical sensitive $Ca^{2+}$ channel (Sukhov et al., 2013), while AP propagation on a voltage-dependent $Ca^{2+}$ (Beilby and Al Khazaaly, 2016; Sukhov et al., 2011). GLR3.3 and GLR3.6, which are associated with initiating SWPs, have not

been shown to be voltage sensitive, and hence cannot be proposed as initiators of APs. This incapacity of extrapolating molecular mechanisms described in SWP to AP and vice versa, have ground on one hand, in the lack of a consensual mechanism for SWP propagation, and on the other, in a lack of knowledge of the functional properties of channels, and calls for further dissection.

There are two models of SWP propagation mechanism: the chemical and the hydraulic, which have theoretical and experimental support but are not easy to reconciliate. The hydraulic theory states that a SWP response can be elicited by a change in the hydraulic pressure inside xylem vessels (Stahlbeth et al., 2006). A pressure increase of 60 kPa in intact pea roots triggers an electric signal that propagates at a rate of 2 – 3 $cm.min^{-1}$ (Stahlberg and Cosgrove, 1997). The fact that the hydraulic wave propagates faster than this signal was experimentally demonstrated (Vodeneev et al., 2012), and fits into theoretical expectations (Sukhov et al., 2013), so it has been hypothesized that the lag between the hydraulic propagation and the electric response happens due to a delayed change of pressure in the electric responsiveness of cells adjacent to the transmitting vessels (Vodeneev et al., 2012). Parallel to this, it is known that the parenchyma cells have a higher elasticity than the vascular tissue,

where the hydraulic wave travels. This means that the former require longer time to change their size to a level that can be sensed by stretch channels, if at all (Huber and Bauerle, 2016). This could account for the delay between the fast transmission of a hydraulic wave in the rigid xylem, and the slower response of the adjacent cells.

On the other hand, the chemical theory of SWP propagation posits that wounding induces diffusion of compounds, released from ruptured cells, along the xylem vessels, and these substances are the ones that elicit an electric response (Sibaoka, 1997). Wound induced diffusion in leaves was measured with luciferin and radioactive $^{14}$C-Sucrose, yielding velocities similar to those of the SWP propagation (Rhodes et al., 1999; Vodeneev et al., 2012). Mathematical modelling demonstrates that the measured diffusion speed of molecules in the xylem is faster than expected in a liquid column. However, it is has been argued that the flow in the xylem is turbulent, facilitating the diffusion of molecules and reconciling the mathematical predictions with experimental observations (Sukhov et al., 2013; Vodeneev et al., 2012).

Additional support to the chemical diffusion theory comes from the application of glutamate (Glu) in very high concentrations (100 mM) on wounded leaves to initiate a propagating signal with the characteristics of a wound-induced SWP (Demidchik et al., 2004; Toyota et al., 2018). These results have been used to postulate Glu as a molecule capable of initiating a SWP and GLR3.3-3.6 as the receptors propitiating it, but the issues implicated with the use of such high concentration are not totally addressed. This molecular mechanism of SWP local excitability deserves further investigation to determine its involvement in the hydraulic and chemical propagation models.

### *Ligand excitability in roots*

Intact roots respond locally to Glu and other amino acids. They present a short depolarization similar to an AP, that is mediated by GLR3.3 (Qi et al., 2006). This AP-like response was recorded in roots completely imbibed in solution, so the whole root was simultaneously stimulated. This makes it impossible to determine if this signal is propagating, and thus remains uncertain whether it is a travelling AP or if it is a local response that corresponds to the initial phase of a SWP. This information is essential to determine the excitable properties

of root cell membranes, the substrate to comprehend the propagation mechanism of electric signals in the tissue.

Moreover, the sensitivity of any tissue to a signal is determined by the receptor gating properties. GLRs activity is optimized by CORNICHON-HOMOLOGUE proteins 1 and 4 (CNIH1-4) which support pollen tube directional growth (Wudick et al., 2018). Such optimization has been observed in heterologous expression of GLRs of the clade 3 with CNIH4, including the electric signaling related GLR3.3 and GLR3.6 (Wudick et al., 2018a)(Simon, et al., under review). It is unknown if CNIHs enhance ligand sensitivity or the electric response in planta, and by extension, the electric responsiveness of plant tissue to Glu.

Comprehension of the Glu-induced electric signal propagation, and GLR gating properties effect on the ligand sensitivity of intact roots can shed light on the molecular mechanisms of SWP, and help outline the building blocks shared by the ancient AP and the novel SWP response. This research aims to characterize the propagation of Glu-induced electric response in roots and determine the role of CNIH4 in it. We hypothesize that the transient electric response of root cells to Glu is a local non-propagating signal, hence

corresponds to the chemical phase of the SWP response observed by mechanical wounding.

## Results

### *L-glutamate (Glu) initiates a local transient signal*

Standardized electric responses were recorded in intact roots after application of Glu. Root cortical cells were impaled at the elongation zone using sharp electrodes (access resistance > 100MΩ), impaling at 5 mm above the tip. Roots were stimulated by 1µl of 1mM glutamate (Glu) (figure 2.1). All the impalements were performed in the second cell layer of the cortical cells i.e., in the second membrane voltage drop below -120mV along the perpendicular progression of the electrode impalement (figure 2.1-A), corresponding to an estimated impalement depth within 15 and 25 µm.

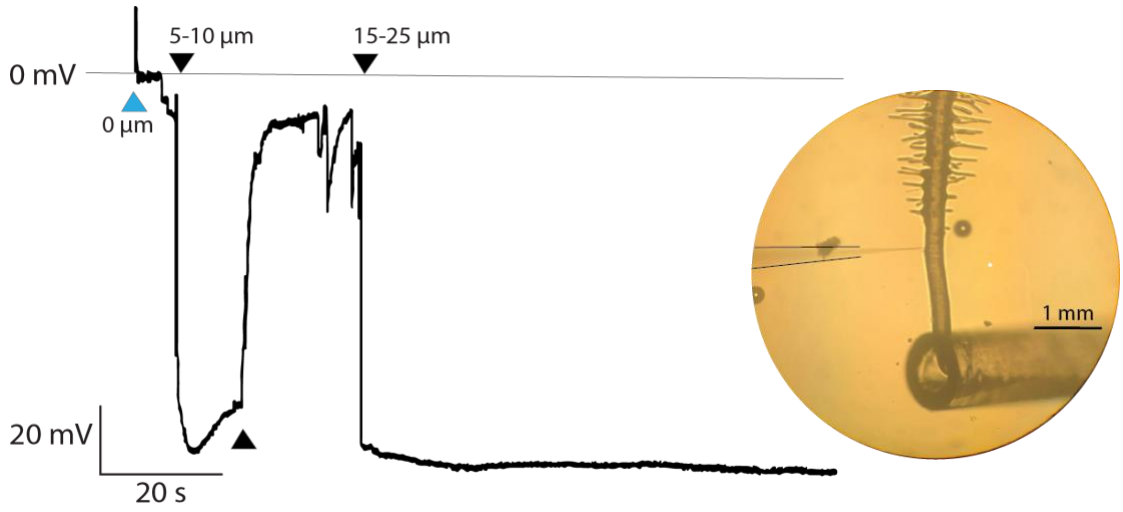



**Figure 2.1**. Experimental setup of root cortical cell impalement with sharp electrodes. Left- Electrical recording of the electrode entering the root tissue. The blue arrowhead denotes contact of the electrode with the root epidermis, subsequent black arrowheads denote transition from extracellular (-20 mV) to intracellular (-120 mV) position. Right- Disposition of the capillary for stimulus application (bottom), and the recording electrode (top – lines illustrate electrode position that approaches in a plane nearly perpendicular to the imaging plane, resulting out-of-focus)

Root cortical cells of wild type plants of *Arabidopsis thaliana* (Col-0) depolarized with an amplitude of 95.6 ± 19.1 mV (mean ± SE, *n*=15) after Glu stimulation (figure 2.2). Simultaneous $Ca^{2+}$ transients were measured with an increase of pixel intensity of 103 ± 29 AU (arbitrary units; *n*=7) from the baseline determined before the stimulus. These $Ca^{2+}$ transients were characterized by a slower raising time, with a half-maximum time of 33 ± 25 s, in contrast to the 2.1 ± 1 s of the electric transient. Subsequently, the membrane potential quickly repolarized, with a half-decay time of 53 ± 38 s after stimulus. The $Ca^{2+}$ transient decayed with a half-time of 148 ± 73 s.

The recorded signal and the stimulus were spatially separated by a distance of over 5 mm, which can be interpreted as reflecting (1) the propagation of the signal, as happens in AP, or (2) the propagation of the stimulus, like in SWP. In an attempt to determine the nature of the propagation a dose-response relationship was established (figure 2.3).

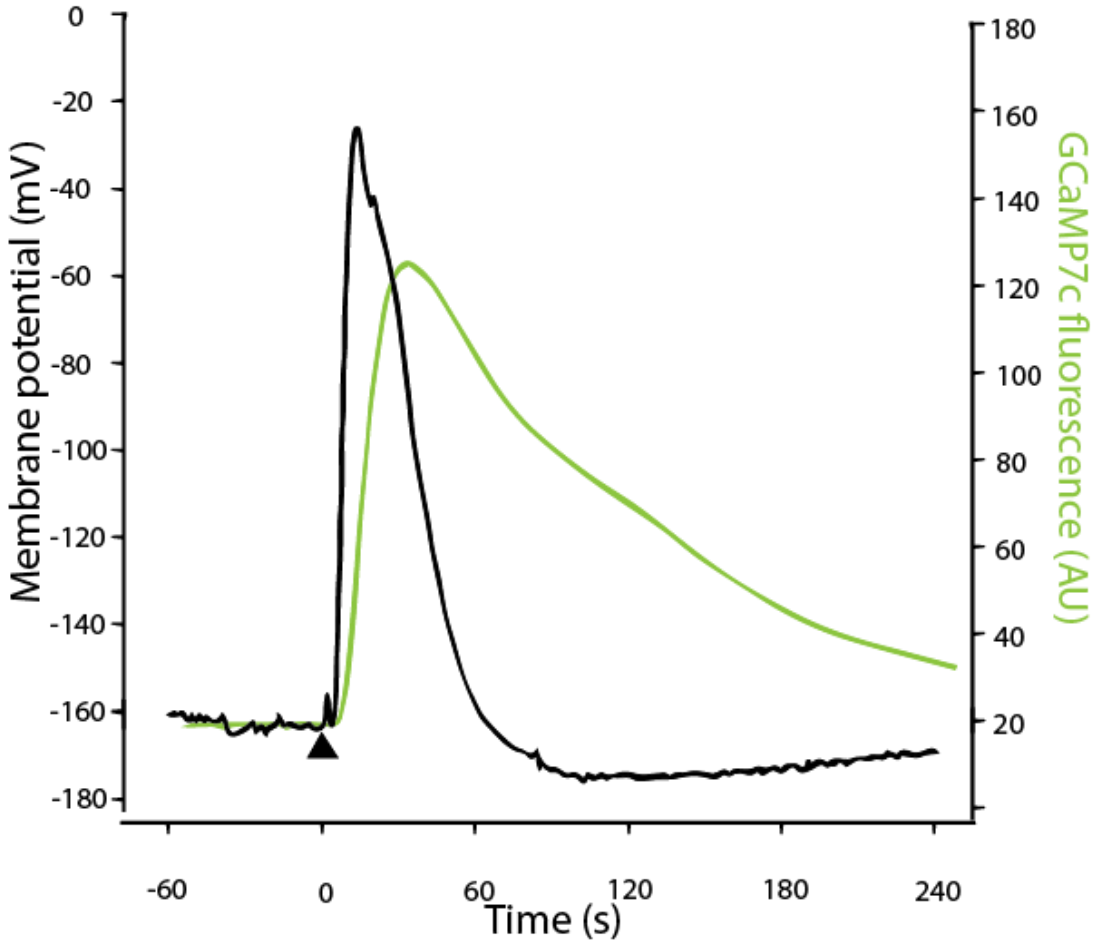



**Figure 2.2.** Electric and $Ca^{2+}$ response elicited by 1mM Glu in root cells. Black trace represents the intracellular membrane potential. Green trace represents the increase of fluorescence intensity of GCaMP7c expressing roots, representative of cytosolic $Ca^{2+}$ concentration.

We observed that the depolarization amplitude increased along with the concentration of Glu. This suggests passive diffusion of the signal rather than an active, electrically coupled propagation proper of an AP. Of relevance, the control stimulation with [Glu] = 0 evoked a depolarization with an amplitude of 45.9 ± 16.5 mV (*n*=9), and the maximum stimulus of [Glu] = 100 mM is moderately smaller 83.8 ± 20 mV (*n*=5) than the response to a ten-fold smaller stimulus 128.2 ± 33.3 mV (*n*=5). Given that stimulation with [Glu] = 1 mM is the minimum concentration at which the curve reached a plateau, the consecutive experiments used a stimulus at that representative concentration.

The passive nature of the propagation could be an internal depolarization that decays over distance, similar to a subthreshold depolarization in an axon (Kandel et al., 2013), or a propagating stimulus that diffuses over the mucilage on root surface, product of adsorption by molecular interactions (Oades, 1978).

To determine if the phenomenon observed is internal decay of an initial depolarization or external propagation of the stimulus, We designed diffusional physical barriers, placed between the recording and stimulus sites. A first approach consisted in a 5 mm thick barrier of agar placed perpendicular

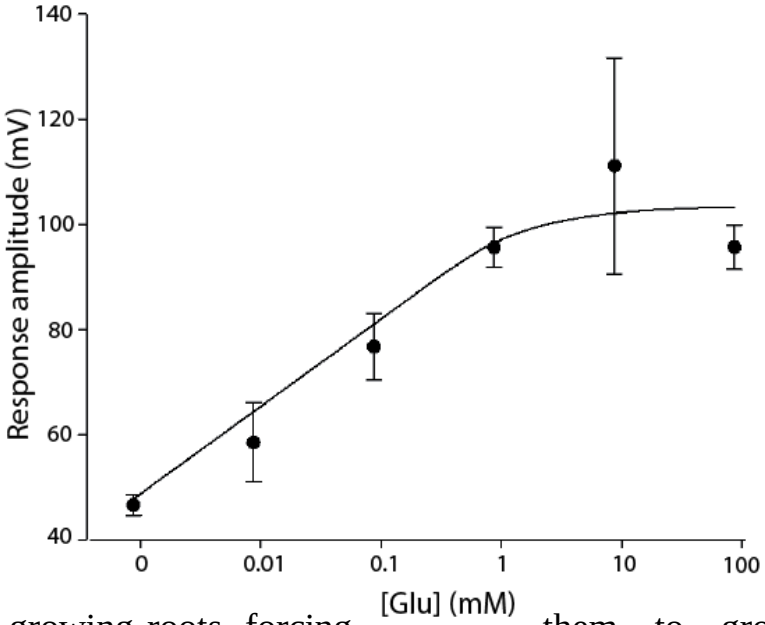



**Figure 2.3**. Dose response curve in impalement of root cortical cells.
Root cortical cells were impaled and stimulated by increasing concentrations of Glu. All the Glu solutions were diluted in 1/2MS pH5.7. The control stimulus at [Glu] = 0 corresponds to 1/2 MS alone.

to the trajectory of the growing roots, forcing them to grow through the agar and emerging on the other side (figure 2.4-A).

This barrier was not intended to prevent glutamate diffusion, since molecules can diffuse in agar with various diffusion coefficients (Schantz and Lauffer, 1962). Instead, this barrier design rather intended to disperse the one-dimensional diffusion that occurred on the surface of the root, into a three-dimensional diffusion inside the agar barrier. Even if Glu diffuses through the agar, given the diffusional half sphere imposed by the agar, concentration would decay with the square of the distance, thus effectively slowing down the concentration wavefront that elicits the depolarization along the longitudinal axis of the root. Such reduction in Glu availability to stimulate

the root should break the signal in case of external diffusion, but any diffusion/reaction internal to the root would remain unaltered.

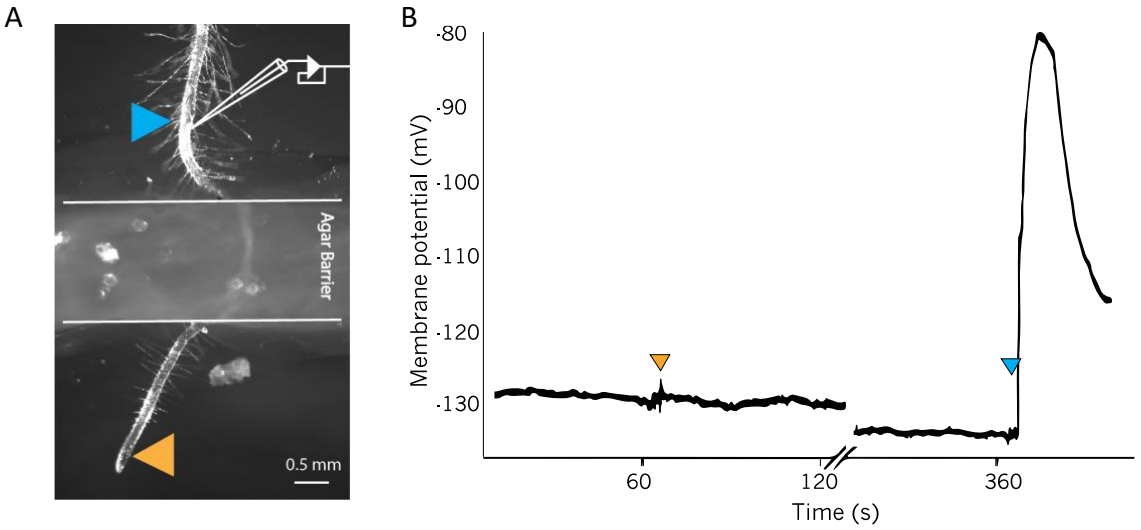



**Figure 2.4**. Membrane potential response to stimuli proximal and distal to the recording site with agar barrier in between.
A. Representation of the experimental setup. A 7-day old root grew through the agar barrier.
B. Intracellular membrane potential was recorded in the mature side of the barrier (electrode diagram-upper part). The root is stimulated with 1 mM Glu distal (orange) and proximal (blue) to the recording site.

Membrane potential was recorded on the upper side of the agar barrier, and 1 mM Glu was applied proximal, and distal to this recording site. The response to the distal stimulus was completely abolished, but to the stimulus on the mature zone, proximal to the recording site, remains as in the control condition, (figure 2.4-B). As a second approach, we aimed at validating these results by using a barrier of mineral oil instead of agar. Given its hydrophobicity, it would be expectable to abolish completely any external diffusion. In accordance with our hypothesis, we observed complete

abolishment of the signal when Glu was applied behind the oil barrier (around 5 mm away). As a control, root tip ablation at the same position where Glu was applied triggered the internally propagating SWP (figure 2.5).

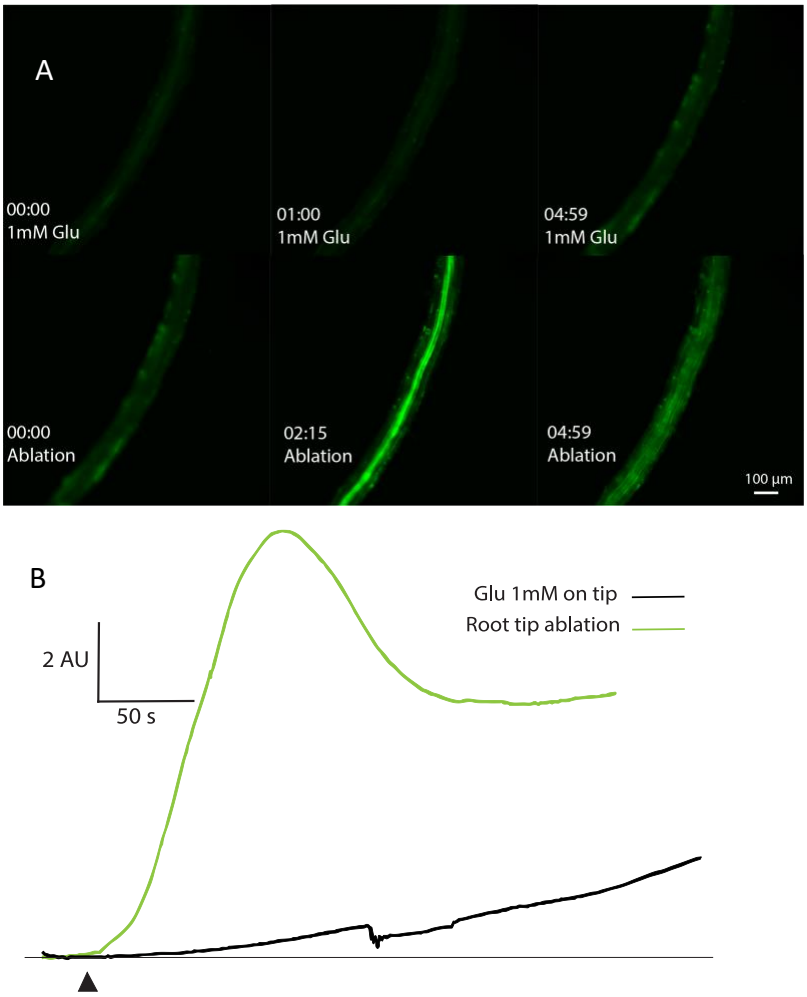



**Figure 2.5**. Recording of intracellular calcium transient of GCaMP7c expressing plant with a mineral oil barrier between stimulus and recording sites.
A. GCaMP7c expressing *Arabidopsis* root. A drop of mineral oil is applied 2-3 mm above the tip, the imaging site is 5-6 mm above the tip. The tip of the root is stimulated with 1mM Glu, and 5 min later it is ablated. The Glu-induced response does not propagate but the internal SWP does.
B. Pixel intensity trace of the time series represented above. Ablation trace is represented by the green line, Glu stimulus by the black line.

The capacity of the stimulus to initiate the stereotypical response only when no barrier stands between the recording site strongly suggests the propagation of the electrical stimulus is in fact a consequence of the diffusion of the externally applied elicitor. This sets the base to argue that the response to Glu

is spatially transmitted by passive diffusion of the stimulus rather than by any internal (excitable) mechanism.

Further repetitions of these experiments were standardized by maintaining a constant distance of 5 mm between the stimulus at the tip and the recording site with or without the diffusion barrier. The difference between the response with and without barrier is significant (figure 2.6).

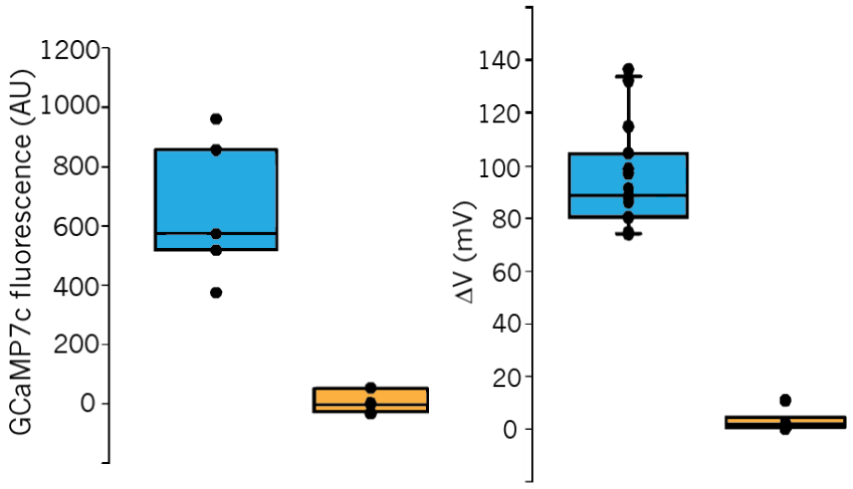
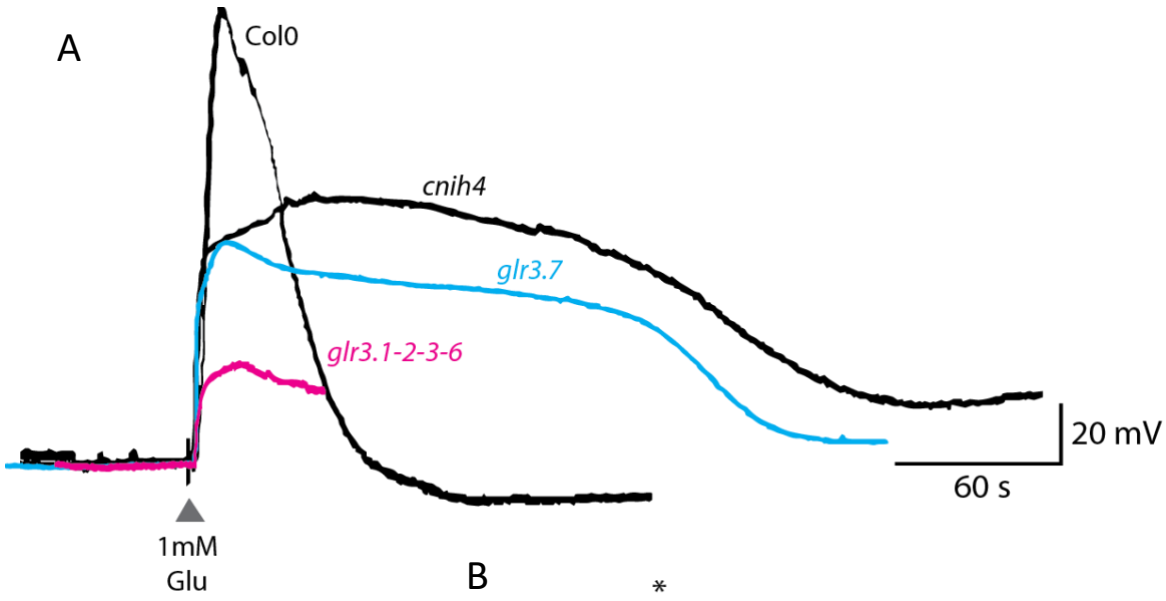



**Figure 2.6**. Effect of agar barrier in long-distance propagation membrane potential depolarizations.
Membrane potential and $Ca^{2+}$ recordings of roots stimulated at the tip, 5mm away from the recording site, under the presence (orange) or absence (blue) of an agar barrier between the recording and stimulation sites.

## *CNIH4 optimize GLR-mediated electric response*

External application of Glu to intact root tissue initiates a local, transient depolarization and cytosolic $Ca^{2+}$ rise. The non-propagating nature of the reported response above is shared with SWP response. We aimed to determine

if the electric and Ca2+ local transients, triggered by Glu and independent of wounding, are sustained by the same molecular mechanisms as the SWP.



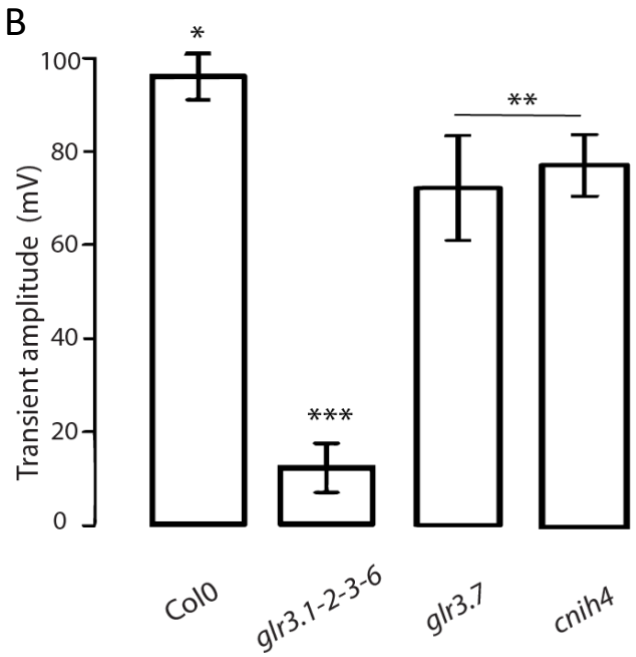




**Figure 2.7**. Amplitude of intracellular membrane potential of *Arabidopsis* wild type and mutant lines.
Single and multiple GLR mutated plants were impaled to record the membrane potential 2 mm away from the tip.
A. Intracellular membrane potential of different genetic backgrounds when 1 mM glutamate was applied.
B. Comparison of amplitude means. All mutant lines are significantly different from the Col0. $p<0.001$ calculated by the Holm-Sidak method. The two GLR mutant signals are not statistically significant between each other. $p>0.001$.

For this we stimulated mutant plants lacking the GLRs that initiate the SWP in response to wounding – GLR3.1-2-3-6.

Roots of mutant plants *glr3.1-2-3-6* were stimulated with 1 mM Glu at the tip and intracellular membrane potential was recorded around 3 mm above the tip with no barrier separating these points. The electric transient recorded presented a significantly diminished response amplitude of 12.0 ± 10.4 mV (*n*=5) (figure 2.7).

Heterologous expression of GLR3.2 and GLR3.3 yield ligand-induced currents only when coexpressed with CNIH4 (Wudick et al., 2018a)(Simon, et al., under review). We thus aimed to determine how that fundamental interaction *in vitro* affects the GLR-mediated electric response herein reported. *cnih4* mutant plants subjected to the same protocol had a mean maximum amplitude after Glu of 78.5 ± 24.8 mV (*n*=14) (Figure 2.8-B), significantly different from that of Col0 plants (t-test $p<0.001$). The response kinetics of the *cnih4* mutant appears to be very different from the wildtype, which a much slower rise after the initial raise, and much prolonged re-polarization. Yet further comparison of the values for half-time raise (*cnih4* - 1.5 ± 0.9 s; Col0 - 2.1 ± 1.6 s; t-test $p=0.972$) and half-time decay (*cnih4* - 55.4 ± 2.8 s; Col0 - 53.5 ± 38.0 s; t-test $p=0.950$) revealed no significant statistical difference.

## Discussion

Our results present evidence that Glu can diffuse externally on the root surface and trigger cytosolic $Ca^{2+}$ increase and membrane depolarization, which is dependent on the activity of various GLRs. The signal is optimized by CNIH4, a promoter of GLR gating capacity in response to amino acids. The external fast diffusion likely occurs in the thin (0.5 μm) layer of mucilage present in intact roots (Oades, 1978), and the reported electric response might correspond to the initial phase of the SWP response.

### *Signal diffusion on root surface*

Excretion of mucilage has been observed on the surface of roots of various plant species. It is composed of multiple polysaccharides and glucans (Oades, 1978). One of the functions of this mucilage is to increase the water availability in the space immediately proximal to the root surface, called the rhizosphere (Ahmed et al., 2014; Schwartz et al., 2015). This stable sheath of liquid medium on the root surface generates a geometry which, in our experimental setup, optimizes diffusion over the small volume determined by the thickness of this layer, thus allowing diffusion parallel to the root

longitudinal axis. This explains the dose-dependent, and distance-dependent response to Glu, and its obliteration by a physical barrier.

There is experimental an theoretical evidence that demonstrates how the liquid sheath formed by mucilage supports nutrient transport along the root surface in the event of drying soil, which could aid the root at distributing nutrient and salt gradients along a bigger area to optimize nutrient absorption and minimize osmotic stress (Ahmed et al., 2014; Zarebanadkouki et al., 2019).

Since multiple signaling peptides have been identified in root secretion of the land plant *Medicago truncatula* (Patel et al., 2018), and given the excitability of root cortical cells in response to Glu here demonstrated, we hypothesize that rhizosphere-directed diffusion can be an effective pathway of signal transmission. Signaling molecules released by any fragment of the root diffuse longitudinally on the root surface, as salts have been shown to do in the rhizosphere (Zarebanadkouki et al., 2019).

*The role of GLR conductance in SWP*

Here we show that Glu can induce a local depolarization and $Ca^{2+}$ increase in root cortical cells. Hence, we propose that a transient, non-propagating, depolarizing response to Glu is inherent to cells located external and internal to the root endodermis. This agrees with the reported depolarization triggered by Glu in protoplast of epidermal cells and in vasculature of wounded roots (Demidchik et al., 2004; Shao et al., 2020).

The Glu-induced electric response of root cells we now report is significantly shorter (53.5 ± 38.0 s, *n*=15) in comparison to the reported wound-induced SWP response (223 ± 125 s, *n*=10) (Nguyen et al., 2018) (t-test p-value < 0.0001). In contrast, the $Ca^{2+}$ transient we report (148 ± 73 s, *n*=7) has a similar duration to the one triggered by wounding (134 ± 36 s, n=4) (Nguyen et al., 2018) (p-values=0.6), or by Glu application to a wounded site (150 s) (Toyota et al., 2018a). Based on these results, we posit that the SWP is the integration of two signaling events mediated by different mechanisms: (1) a fast (1-2 s) peak depolarization and a $Ca^{2+}$ transient increase that decays after 150 s and is independent of wounding, and (2) a sustained depolarization and increased $Ca^{2+}$ cytosolic concentration that lasts several minutes and is dependent on wounding (Nguyen et al., 2018).

The depolarization of roots upon Glu application is abolished in the *glr3.1-2-3-6* plants. Currents triggered by amino acid application in root protoplast, recoded using patch clamp, were also diminished in the *glr3.1-2* double knockout, furtherly diminished in *glr3.3*, and completely abolished in *glr3.6* (Mou et al., 2020). This leads to the conclusion that GLR3.6 is the directly implicated in the signal triggered by Glu in intact roots, while GLR3.1-2-3 are only partially implicated.

Our data also suggests that the initial fast depolarization of SWP corresponds to the transient we now describe. Both are mainly mediated by GLR3.3-6, and their depolarization peak and $Ca^{2+}$ transient have compatible kinetics (Mou et al., 2020; Nguyen et al., 2018; Toyota et al., 2018b). This signal is local, but in the case of SWP, the ligand is likely propagated by diffusion through the xylem, and accelerated by its turbulent flow (Sukhov et al., 2013; Vodeneev et al., 2012).

Despite the fact that GLRs are implicated in this local depolarization and cytosolic $Ca^{2+}$ increase, it is known that GLRs could permeate anions as well as cations. Then arises the question of whether these receptors mediate $Ca^{2+}$

currents or anionic currents in the ligand-triggered response. In the case of GLR $Ca^{2+}$ permeability, they would account for the $Ca^{2+}$ transient, but would require the activity of hyperpolarizing channels to accomplish a depolarization shorter than the $Ca^{2+}$ increase. On the contrary, in the case of anionic permeability, it can be argued that GLRs account for the initial depolarization and activate a $Ca^{2+}$ conductance. This questions requires further research that determines the pore selectivity of the specific heterotetramers present in excitable cells.

In this research we present a depolarization response of 40 mV initiated by application of control solution in the absence of ligand (1/2MS, pH5.7). Such depolarization was also observed in the quadruple mutants *glr3.1-2-3-6*, suggesting that it is likely not mediated by GLRs. Moreover, the regression of the dose-response curve showed a best fit to a 4-parameter hill equation, which should have a sigmoidal shape that was not fully obtained. This is due to the existence of a response that is not related to amino acid stimulus; hence the initial flat phase of the ligand-related response sigmoidal curve is masked by the response to another stimulus.

It is possible that this non ligand-related depolarization is triggered by the mechanical artifact of the stimulation mechanism, which could induce depolarization by anionic efflux through mechanosensitive channels (Guerringue et al., 2018). MSL10 has been shown to mediate chloride currents in heterologous expression and, along other members of the MSL family, underpin stretch-activated currents in root protoplast (Haswell et al., 2008). MSL channels could be responsible not only for the response we report but could actually have a function as the initiators of the hydraulic component of SWPs.

Furthermore, it has been hypothesized from mathematical models that sustained depolarization in SWP, the second phase, is product of proton pumps inactivation by the influx of $Ca^{2+}$ through ligand-gated $Ca^{2+}$ channels (Sukhov et al., 2013). This agrees with the abolition of the two phases of SWP in *glr3.3-6* (Nguyen et al., 2018). However, this hypothesis is not compatible with the transient signal we now report, in which intracellular $Ca^{2}$ increases but does not induce a sustained depolarization, suggesting that the occurrence of two signals is required for this sustained depolarization to occur.

It is possible that in the SWP response the proton pump inactivation requires the occurrence of the initial ligand-gated $Ca^{2+}$ transient, and additionally, other events likely related to the hydraulic wave. It is also possible that only the cell types adjacent to the xylem, but not the cortical cells, present a mechanism of $Ca^{2+}$-dependent proton pump inactivation, hence respond differently to the same ligand-induced transient. Further research on the mechanism that controls sustained depolarization in SWP is necessary to understand how information transduction of wounding events occurs in plants, and how electric signals in higher plants evolved as a synergy of simpler signaling mechanisms.

## Methods

### *Plant material*

*Arabidopsis thaliana* Col-0, *glr3.7* (Salk_103942), and *cnih4* (Salk_145991) lines were obtained from the Salk institute (La Jolla, CA, USA). *glr3.1-2-3-6* was developed and obtained from Edward Farmer (University of Lausanne, Switzerland). Col-0 plants were transformed with the bud dipping method in

*Agrobacterium* containing plasmid vector with GCaMP7 controlled by 35S promoter.

Seeds were surface sterilized and sown on petri dished 0.8% agar with half strength Murashige-Skoog salt, and then stratified in 4º C for 24 hours. The plates were then subjected to a light/dark regime of 16:8 at 20º C in a vertical position to allow roots to grow in a linear manner. 7-10 days old plants were used for data acquisition.

*Root cell impalement*

For current-clamp recordings, roots were hydrated 20 minutes prior to impalement with liquid 1/2MS pH 5.7. The dishes were placed on an inverted microscope and the roots were impaled at ≈2mm above the root tip and 2-3 cells (10-40μm) deep into the tissue. The impalement was done with a sharp electrode, resistance >100MΩ, filled with 3M KCl. Pipettes were pulled using a P97 puller (Sutter Instrument, Novato, CA, USA). The membrane voltage was tracked at a sampling frequency of 2-4 kHz and filtered at 1-2 kHz using an Axopatch 200A amplifier with an Axon 1200 DigiData analog-to-digital converter and pCLAMP software (Molecular Devices, Sunnyvale, CA, USA).

For the stimulus of the tissue a 1µl drop was delivered with a 1mm thick capillary placed at the tip of the root and controlled by a CellTram microinjector (Eppendorf AG, Hamburg, Germany). The D-mannitol and L-Glu stimulus solutions (Sigma-Aldrich, St. Louis, MO, USA) were made at a concentration of 1mM on liquid 1/2MS and balanced to pH5.7.

### *Microscopy and Ca2+ imaging*

Fluorescent observation of GCaMP7c expressing plants was done using an MVX-ZB10 Olympus stereozoom (Olympus America Inc., MA, USA). Illumination was provided by a MVX coaxial fluorescent illuminator with a GFP filter set with excitation: 470/40 nm, dichroic: 495 nm long-pass, emission: 525/50 nm. Image acquisition was done with a Hamamatsu Flash4.0 V3 sCMOS digital camera. The acquisition of time series was standardized at exposure time of 250 ms, laser at 25% intensity, and sample rate 1Hz.

### *Data processing and analysis*

Image processing and quantification was done using FIJI. All data resulting from electrophysiological recordings and fluorescent imaging was processed in R, and statistical tests were done in SigmaPlot (Systat, San Jose, CA, USA).

# Chapter 3: GLR3.7 mediates root hair elongation by maintenance of apical depolarization

## Introduction

Root hairs are unicellular, tubular projections specialized in water and mineral uptake, soil anchorage and microbe interaction (Eshel and Beeckman, 2013). In *Arabidopsis*, root hairs have a diameter of about 10μm and can elongate up to 1mm from the root epidermis (Grierson et al., 2014). Root hairs originate from differentiated epidermal cells, the trichoblasts. The radial distribution pattern of trichoblasts and atrichoblasts in the root occurs in a stereotypical, controlled manner along the epidermis that results in 18 to 22 files of trichoblasts. Elongation of root hairs is driven by conserved mechanisms that relays on proper homeostasis of $Ca^{2+}$ increase and ion fluxes at the cell tip (Becker et al., 2014; Schiefelbein et al., 1992; Vissenberg et al., 2001).

### *Trichoblast differentiation*

On radial plane, the epidermis of *Arabidopsis* roots is formed by 18 to 22 files of cells (Berger et al., 1998), along which trichoblasts are equally separated from each other by atrichoblasts. This is determined by a strict position-

dependent pattern (Galway et al., 1994). The cell layer underneath the epidermis is the cortical cell layer. The fate of epidermal cells is determined by their relative position over the underlying cortical cells: if an epidermal cell is in contact with two cells of the cortical layer, it differentiate into a trichoblasts, if it is in contact with only one cortical cell, stays as an atrichoblast (figure 3.1). This condition, and the geometrical disposition of root cells, assures that trichoblasts are always separated by one or two atrichoblasts in the circumference of the root (Dolan et al., 1994; Galway et al., 1994).

The pattern of genetic transcription differs between root hair forming and non-forming cells. Atrichoblasts, specifically, express the genes *GLABRA2* (*GL2*), *WEREWOLF* (*WER*), and *TRANSPARENT TESTA GLABRA* (*TTG*) (Galway et al., 1994; Lee and Schiefelbein, 1999; Masucci and Schiefelbein, 1996). Interestingly, the earliest expression of *GL2* occurs prior to the formation of the meristem, which primes the initial positions that later cues the position-dependent pattern as the root grows (Lin and Schiefelbein, 2001).

In contrast, trichoblasts have clear morphological differences before the clear bulging of the root hair, including faster cell division rate, reduced cell length,

and unique cell surface ornamentation (Dolan et al., 1994). Also, trichoblast formation is promoted by the phytohormones ethylene and auxin.

Application of ACC, precursor of ethylene, induces formation of ectopic root hairs (Tanimoto et al., 1995), and mutations in genes of the ethylene and auxin signaling pathways generate roots without hairs, as for example, *AUXIN RESISTANT2* and *3* (*AXR2*, *AXR3*) (Leyser et al., 1996); and *ETHYLENE INSENSITIVE 2* and *3* (*EIN2*, *EIN3*), members of the ethylene signaling pathway (Feng et al., 2018, 2017; Martín-Rejano et al., 2011).

Other genes identified as regulators downstream of auxin and ethylene pathways are the *ROOT HAIR DEFECTIVE 6* (*RHD6*), and members of its family *ROOT HAIR DEFECTIVE 6-like* (*RSL*). Mutations in these genes induces hairless roots, and can be recovered by application of ACC or the auxin indole-3-aceti acid (IAA) (Feng et al., 2017; Honkanen and Dolan, 2016; Masucci and Schiefelbein, 1996; Proust et al., 2016).

The genes that determine each cell type interact across neighboring cells, giving rise to the radial distribution pattern described: The activity of *GL2*, *GL3*, and *WER*, is endogenous to every cell and inhibits *RHD6*, hence cells

stay an atrichoblast as a default fate. On the other hand, the gene *SCRAMBLED* (*SCM*), that accumulates from the interaction with cortical cells, inhibits the activity of *WER*, allowing *RHD6* to function, thus diverting from the default epidermal cell fate and inducing trichoblast formation through the activity of ethylene and auxin (figure 3.2) (Bruex et al., 2012; Kwak and Schiefelbein, 2008).

### *Root hair initiation*

An epidermal cell that differentiates into a trichoblast undergoes physiological and morphological changes that allows it to locally swell on the basal end and elongate the cell wall and membrane on the apical growing end to form the root hair. Initially, GTPases are recruited to the region that is going to bulge by the receptor-like kinase FERONIA (FER) (Duan et al., 2010). In parallel, the pH of the cell wall is lowered, and the cytoplasmic pH elevates from 7.3 to 7.7 at the initiation site (Bibikova et al., 1998). Acidification of the apoplast likely activates expansins that also accumulate in this area. These proteins loosen the cell wall to allow expansion (Bruex et al., 2012), culminating in the generation of a bulge in the membrane, inside which actin filaments are accumulated (Baluška et al., 2000). All this process is directed by the

expression of *RSL4*, which prolongs shortly during the elongation phase, and determines the final length of the root hair (Datta et al., 2015; Lee and Schiefelbein, 2002).

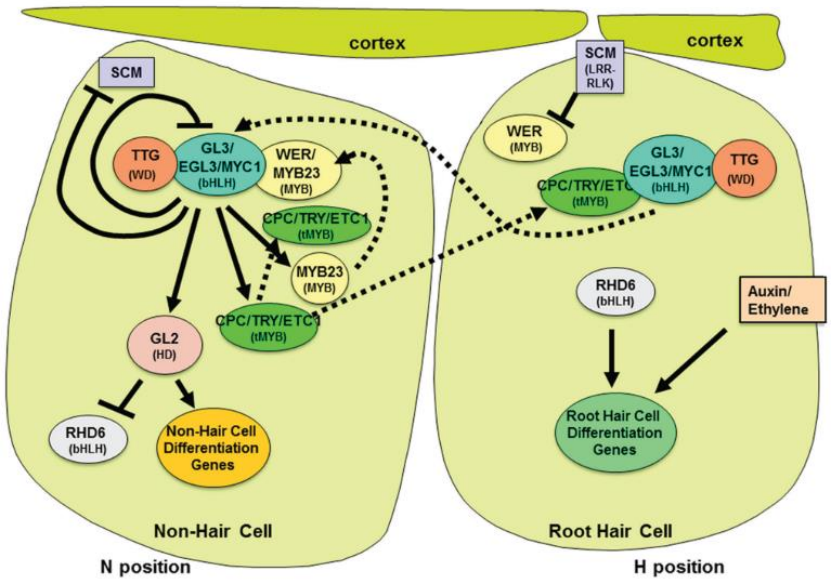



**Figure 3.2**. Regulatory feedbacks between cortical cells atrichoblasts (N) and trichoblasts (H). SCM coming from cortical cells inhibits the positive feedbacks of atrichoblast determining genes, that allows RHD6 to initiate trichoblast differentiation. It is no known that other RSL genes are affected but are not included in this figure Proust et al., 2016. Modified from Grierson et al., 2014

The initiation process is a transition towards implementing the necessary machinery that allows tip elongation. This machinery involves the expression of the RSL gene family, which is conserved across land plants, and ionic channels that support strong ionic currents, and constant cytoplasmic streaming that provides vesicles to the elongating tip (Emons and Ketelaar, 2009; Proust et al., 2016).

*Root hair elongation*

The elongation phase is characterized by tip elongation at a pace of 1-2μm.min$^{-1}$, which requires constant exocytosis of vesicles loaded with the necessary material for cell wall synthesis and membrane deposition (Ketelaar et al., 2008). Simultaneously, it is necessary to maintain a positive internal pressure that assures expansion, and constant cytoplasmic streaming that provide vesicles to the root tip. This is sustained by constant ionic fluxes that drive water into the cell and also organize the cytoplasm, and actin deposition that composes the path for cytoplasmic streaming (Emons and Ketelaar, 2009).

At this stage, the growing tip is defined by the peak of a cytoplasmic $Ca^{2+}$ concentration gradient, which determines the direction of growth, and disappears when the root hair stops elongating (Schiefelbein et al., 1992; Wymer et al., 1997). The function of this important signaling ion at the growing tip is to promote vesicle fusion, and actin cytoskeleton turnover to allow flexible expansion, while the acid pH and the GTP-binding proteins at the tip are maintained (Bibikova et al., 1997; Ketelaar, 2013; Pei et al., 2012).

Both elongation and $Ca^{2+}$ have an oscillatory behavior. The $Ca^{2+}$ gradient fluctuates with two main frequency components: a low frequency around 0.01Hz, and a high frequency around 0.04Hz, the latter is coordinated with oscillations of cytoplasmic pH, ROS production, and elongation frequency (Candeo et al., 2017; Lassig et al., 2014; Mendrinna and Persson, 2015). Interestingly, $Ca^{2+}$ oscillation in moss apical elongating cells also coordinate the turnover of actin polymerization with cell growth (Bascom et al., 2018).

The membrane potential of root hairs is maintained between -160 and -200mV, which ensures extracellular influx of $Ca^{2}$, and influences the speed of cytoplasmic streaming (Ayling et al., 1994). Hyperpolarization-activated $Ca^{2+}$ channels, and Depolarization-Activated $Ca^{2+}$ Channels have been identified in protoplasted root hair tips (spheroblasts) (Miedema et al., 2008; Véry and Davies, 2000). In *Arabidopsis* pollen tube, members of the GLRs and Cyclic Nucleotide-Gated Channels (CNGC) families have been identified as the $Ca^{2+}$ conductances that sustain the ionic gradient and growth (Frietsch et al., 2007; Michard et al., 2011). While CNGCs can regulate intracellular $Ca^{2+}$ oscillation, and some have been characterized as voltage-dependent (Dietrich

et al., 2020), no genetic identity is known for the $Ca^{2+}$ channels implicated in root hair elongation.

It is known that environmental chemical cues can alter root hair development. E.g., low phosphate or boron concentrations promote root hair formation and elongation. Nitrogen is another important nutrient that roots can only take in as organic compounds, like amino acids. There is no evidence that links root hair development with amino acids, but these can initiate electric and $Ca^{2+}$ transients in root cells (Mou et al., 2020; Qi et al., 2006). A similar transient response is triggered by wounding, and has also been observed to increase ethylene (Marhavý et al., 2019). These fragments of data prompted us to consider a novel hypothesis for the mechanism beyond root hair development, implying a link between amino acid electric sensitivity, $Ca^{2+}$ increase, and ethylene production. On the other hand, recent evidence converges to suggest that GLRs are implicated in one or more stages of root hair development: exogenous chemical cues can alter root hair development (Martín-Rejano et al., 2011; Savage et al., 2013); root cells can sense exogenous amino acids, including ACC, and respond by membrane depolarization and intracellular $Ca^{2+}$ increase mediated by GLR3.3/3.6 and in a less degree by GLR3.1/3.2 (Mou et al., 2020; Stephens et al., 2008); not only ACC is precursor of

ethylene, but also electric and $Ca^{2+}$ transients induce ethylene production (Marhavý et al., 2019), necessary for root hair development; lastly, GLR1.2 and GLR3.7 ensure proper $Ca^{2+}$ homeostasis and pollen tube elongation in *Arabidopsis* (Michard et al., 2011).

The presence of all the 20 GLRs in the ensemble of all the different root cells (Chiu et al., 2002) increases possible functional redundancies, hence compensating the lack of one functional channel with others, notwithstanding functional combinations by heteromerization. This is a challenge for determining GLR function by reverse genetics. Furthermore, novel information on electrophysiological properties of the GLR clade 3 from our group (Simon et al., under review) seems to implicate members of this clade as likely implicated in ligand gating functions. Of relevance, GLR3.7 enhances the ligand sensitivity and current intensities of other GLRs when heteromerized together (Simon et.al., under review). We hypothesize that strongly gated GLR3.7, most likely heteromerized to other clade 3 members, support the $Ca^{2+}$ homeostasis during root hair elongation, hence maintaining proper apical growth.

## Results

### *GLR3.7 and Glu induce root hair elongation*

In wild type and mutant glr3.7 plants, the amount and length of root hairs was determined in a section of 5 mm in the mature zone of the root, where root hairs growth ceased (Datta et al., 2015). The count of elongated trichoblasts was not significantly different between Col-0 (49.9±12.8, n=275) and glr3.7 (50.2±10.8, n=30). However, the length of those root hairs was significantly smaller by ca. 1/3 in the mutant (0.12±0.05mm) in comparison to Col-0 (0.32±0.16 mm; p-value<0.01) (figure 3.3).

As demonstrated in chapter 2 of this thesis, the application of Glu induces a transient depolarization and Ca2+ increase in root cells, which is diminished in the *glr3.7* mutant. Additionally, similar electric transients have been shown in response to other proteinogenic amino acids (Stephens et al., 2008). This led to the hypothesis that other amino acids might influence root hair development through GLR activation.

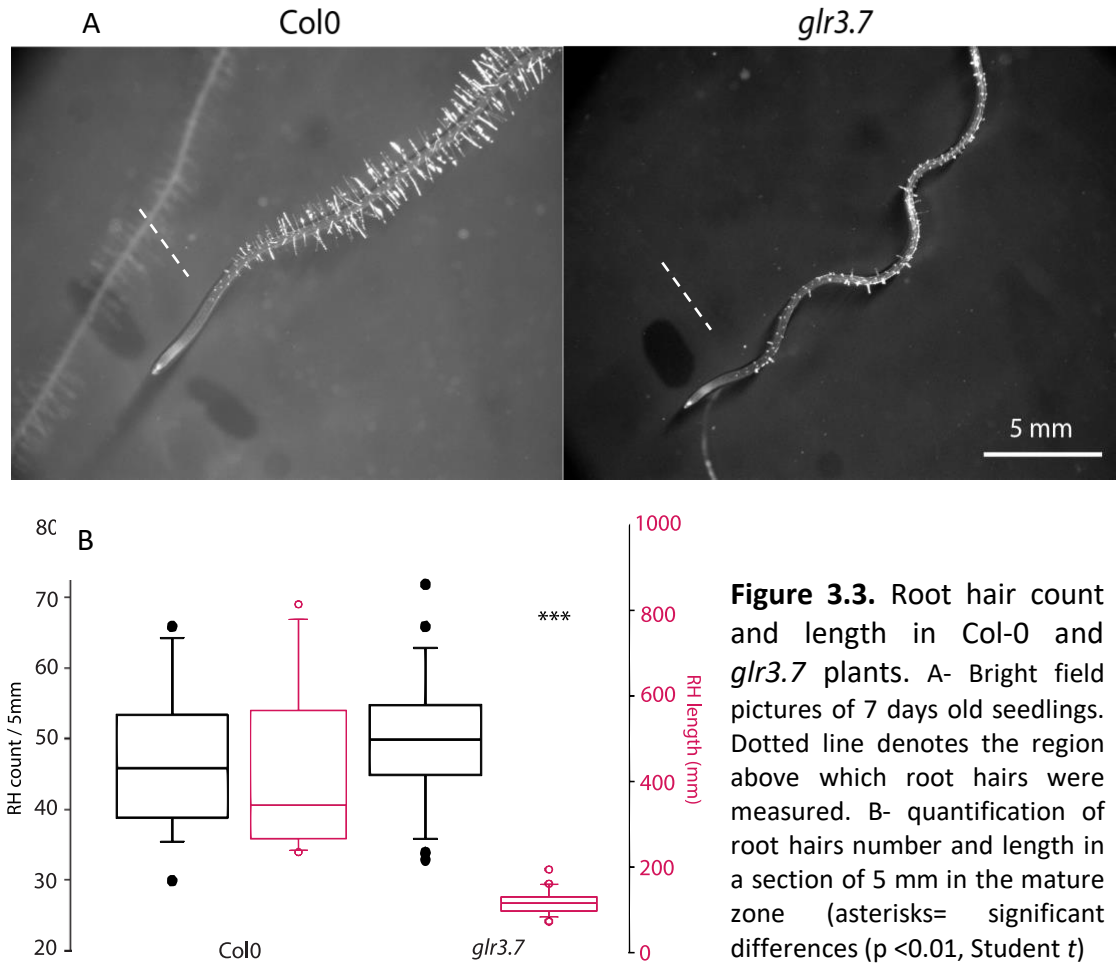



**Figure 3.3.** Root hair count and length in Col-0 and *glr3.7* plants. A- Bright field pictures of 7 days old seedlings. Dotted line denotes the region above which root hairs were measured. B- quantification of root hairs number and length in a section of 5 mm in the mature zone (asterisks= significant differences (p <0.01, Student *t*)

To characterize their influence on trichoblasts differentiation and elongation of root hairs, we screened ACC, Glu and other proteinogenic amino acids described in the literature as having any stimulation of electric activity compatible with GLR properties. We thus challenged roots with asparagine (Asp), cysteine (Cys), methionine (Met), L- and D-Serine (Ser) and Glu, all diluted in liquid 1/2MS growing medium to the final concentrations of 0.1 and 1.0 mM. 5µl of solution was applied on the root tip, and after 24 hours, where

the root continued to grow, all visible root hairs were counted and measured from tip to the base at the epidermis.

All the proteinogenic amino acids induced a response that clustered between the strongest effect of ACC (1.0 mM) (count:81.7±10.7; length:1.09±0.19mm, *n*=13; see upper right corner of the graph, detached from all others) and the 1/2MS control treatment (count:49.9±12.9; length:0.52±0.22mm, *n*=14) (figure 3.4). The control treatment was not statistically different both in terms of count and length from Col-0 without treatment. The root hair response for both concentrations of all the treatments did not differ strongly, with the only exception of methionine at 0.1mM, that caused a decrease of count and length. Glu in both concentrations induced the strongest root hair elongation (Glu 0.1mM-length:0.66±0.08mm, *n*=10) (Glu1mM- length:0.72±0.14mm, *n*=10). Given that Glu (1mM) yields the longest root hairs among the proteinogenic amino acids screened, it is further used to investigate how amino acids induce elongation.

Having characterized the response to amino acids, we aimed to determine if the enhanced elongation obtained from Glu is mediated by GLR3.7, and the possibility of multiple GLR being partially involved. This shared function

happens in Glu-triggered electric signaling (Nguyen et al., 2018), or in ACC sensing by GLRs in Arabidopsis root protoplasts (Mou et al., 2020), where GLR3.1, GLR3.2, GLR3.3 and GLR3.6 share the role of ligand-dependent response mediators. To do this, stimulation with 1mM Glu and ACC was done

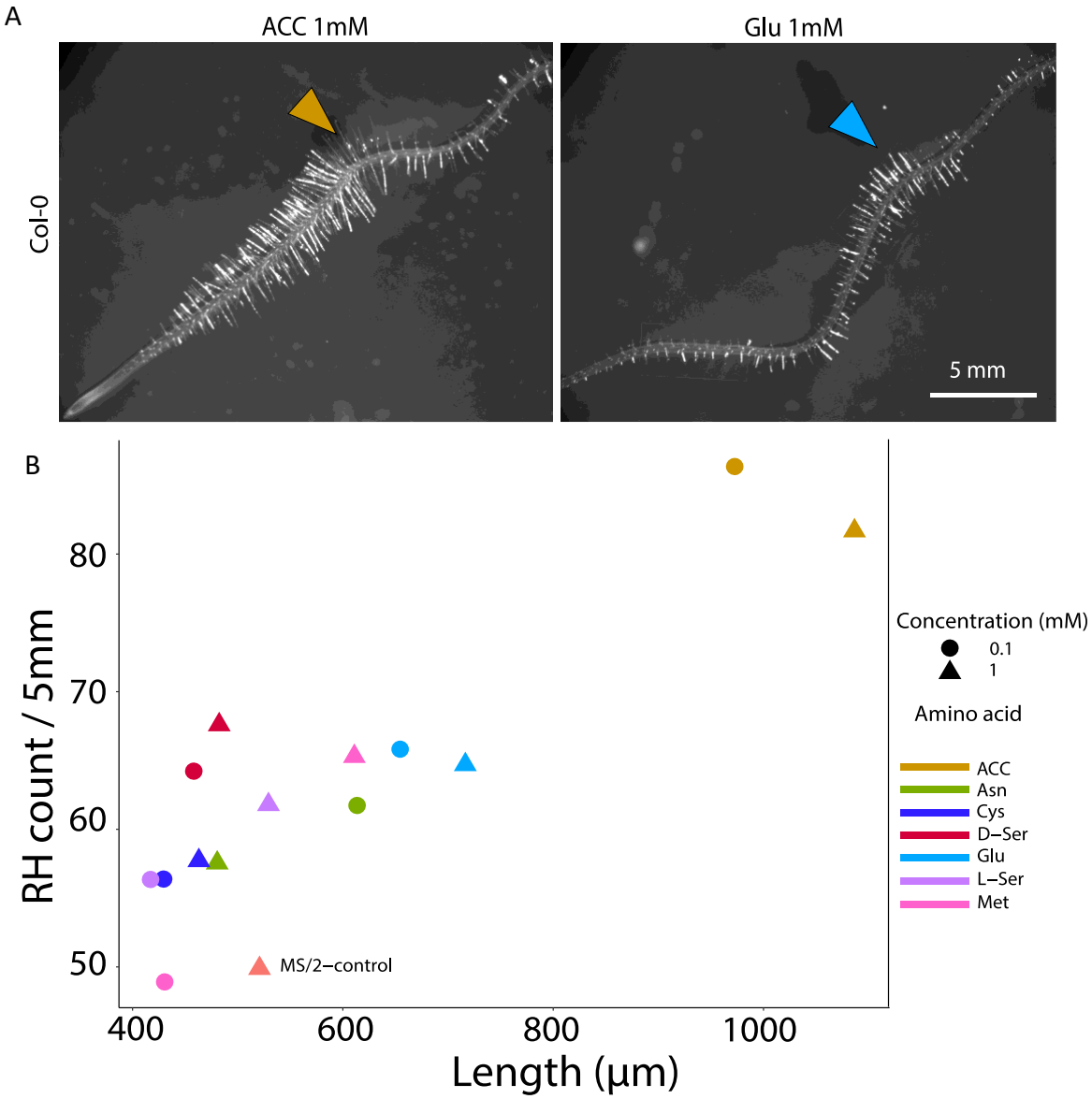



**Figure 3.4.** A- Bright field pictures of 7 days old seedlings stimulated with 1mM ACC and Glu. Arrowhead denote application site. Measurements were done 5mm below this point. B- Root hair count and length in Col-0 after application of various amino acids.

in root tips of mutant plant with GLRs knocked out by insertion. The candidates chosen were *glr3.1-2-3-6,* for the previously exposed roles.

Various GLR KOs treated with Glu showed an equal or even greater increase of root hair count and length than the wild type. The mutant *glr3.1-2-3-6* (count:77.6±10.9; length:1.13±0.11mm, $n$=13) presented a significant increase in count and elongation ($p<0.05$). The increased response of GLR mutants to Glu is counter-intuitive, but it could be explained by compensating over-expression of other GLRs in response to the unbalanced transcription network of the mutants. In contrast, *glr3.7* was the only GLR mutant to have a significant decrease in root hair length, while the count was not altered in comparison to wildtype plants (figure3.5).

*Specific functions of GLR3.7 and Ethylene/EIN2 in root hair elongation*

ACC increased the amount of root hairs counted and their length (figure 3.4), which agrees with the ethylene induction of trichoblast differentiation, and elongation promotion through the ethylene receptors (Martín-Rejano et al., 2011; Pitts et al., 1998). Also, the application of amino acids increased the

amount of root hairs counted. This makes evident a functional overlap of different signals in the differentiation and elongation processes.

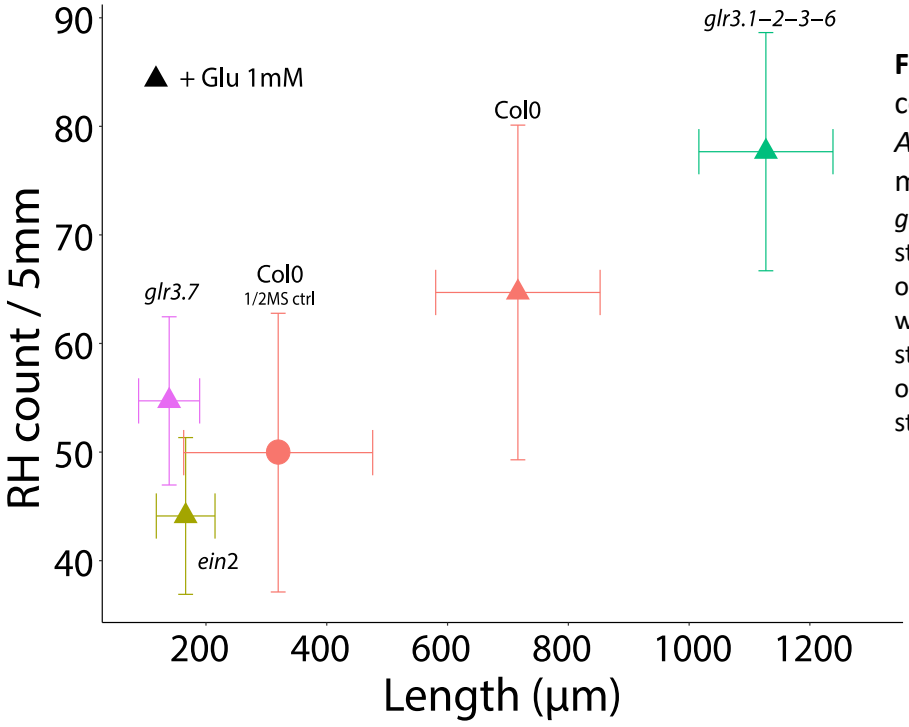



**Figure 3.5.** Root hair count and length in *Arabidopsis* mutants. *Glr3.1-2-3-6, glr3.7* and *ein2* mutants stimulated with a drop of 1mM Glu. Root hairs were counted 24h after stimulation in a section of 5mm below the stimulation site.

In order to resolve the functions of GLR3.7 and EIN2, we observed responses at all the combinations of *glr3.7* and *ein2* stimulated with ACC and Glu (1.0mM). It was clear that GLR3.7 only underlies elongation in responds to Glu and ACC. On the other hand, EIN2 underlies differentiation and, partially, elongation in response to ACC only (figure 3.6-A).

Trichoblast differentiation is mediated by EIN2. After treatment with ACC, there was an increase of root hairs in Col-0 (74.3±15.3, *n*=10) and *glr3.7*

(63.6±15.6, *n*=15), but not in *ein2* (45.1±6.1, *n*=10) (figure 3.6). In contrast, the Glu stimulation enhanced the root hair count in Col-0 (64.7±15.4) only, but not in *ein2* (44.1±7.2, *n*=9) nor in *glr3.7* (54.7±7.8, *n*=14). It was observed that the increased root hair count in Col-0 roots treated with Glu raised from the quantification of short root hairs that were not visible in untreated roots but are long enough to be counted in treated roots. Thus, increasing the count in Col-0 but not in *glr3.7*, in which root hairs did not elongate to be counted.

Root hair elongation is impaired in *glr3.7* (1/2MS: 0.13±0.05mm, *n*=14; Glu:0.14±0.05mm, *n*=14; ACC:0.13±0.04mm, n=15). Similarly, in *ein2* root hairs are also shorter (1/2MS:0.16±0.05mm, *n*=15), and do not increase when treated with Glu (0.17±0.05mm, *n*=14), but increase after ACC application (0.22±0.09mm, *n*=10). However, ACC-induced elongation is not enough to recover the length of wildtype root hairs. Then, root hair elongation is directed by GLR3.7, enhanced by exogenous application of either Glu or ACC, but requires the integration of EIN2 activity to fully rescue the wildtype phenotype.

GLR3.7 may regulate elongation through membrane voltage depolarization

Apical growth is characterized by the existence of an apical gradient of $Ca^{2+}$, with increase concentration at the root hair tip (Wymer et al., 1997). This gradient is believed to relay on membrane voltage maintenance (Mendrinna and Persson, 2015). Since GLR3.7 and EIN2 influence root hair elongation,

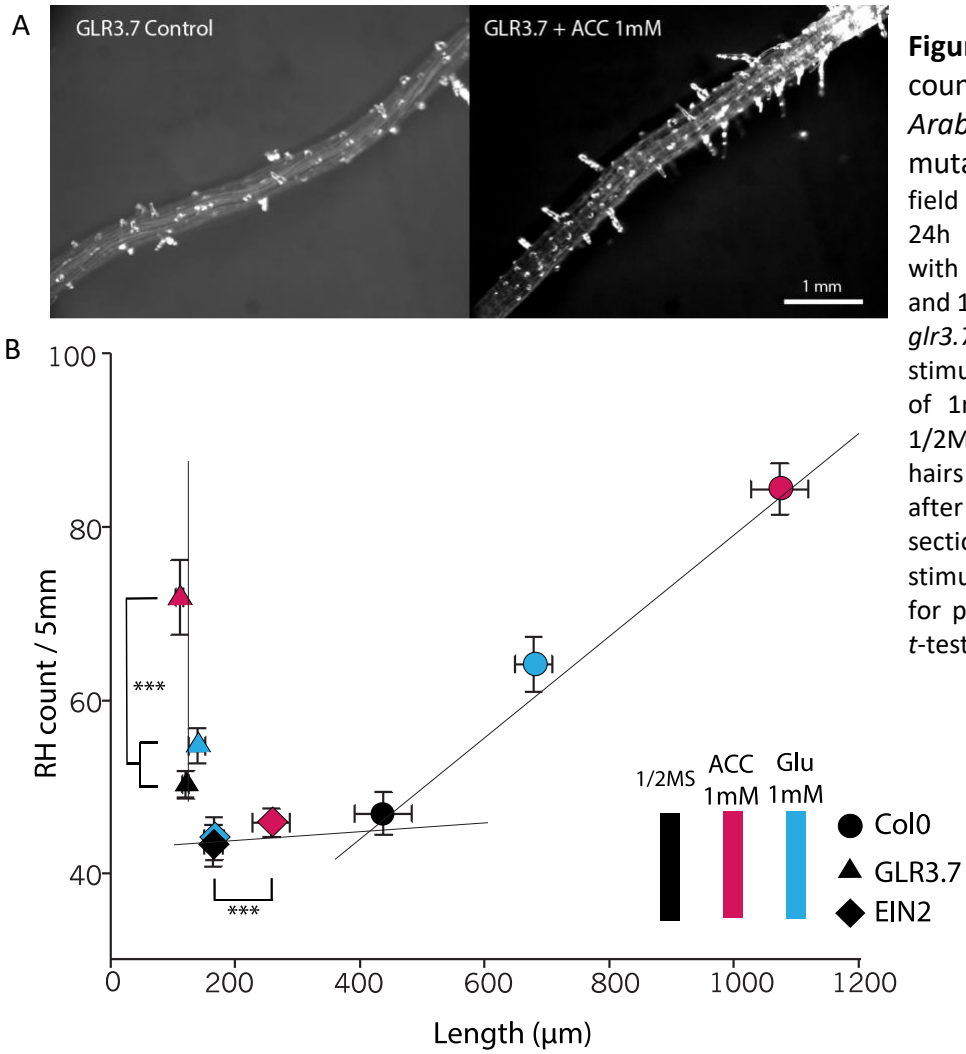



**Figure 3.6.** Root hair count and length in *Arabidopsis* mutants. A- Bright field image of *glr3.7* 24h after treatment with negative control and 1mM ACC. B- Col-0, *glr3.7* and *ein2* mutants stimulated with a drop of 1mM Glu, ACC, or 1/2MS as control. Root hairs were counted 24h after stimulation in a section 5 mm below the stimulation site. (*** for p-<0.005, Student's *t*-test.)

we attempted to determine if both receptors influenced the $Ca^{2+}$ and electric

aspects of elongation. For this we determined the membrane potential of elongating root hairs of *glr3.7*, *ein2*, and Col-0 plants.

Seedlings were incubated in a voltage-sensitive fluorescent dye, Fluovolt, which yields a signal that increases upon membrane depolarization. This technic successfully showed a tip localized depolarization in elongating Col-0 root hairs (figure 3.7-A). The fluorescence at the tip, normalized by subtracting the basal fluorescence of atrichoblasts, was quantified in growing and non-growing root hairs (figure 3.7-B). In Col-0, the fluorescence intensity of growing root hairs (43.36±32.53 AU, *n*=94) was significantly higher in comparison to mature, non-growing ones (10.84±29.82 AU, *n*=79). In *ein2* plants, growing root hairs were also more depolarized (66.93±42.14 AU, *n*=38) than mature root hairs (-10.84±34.36 AU, *n*=62).

In contrast, all the root hairs of *glr3.7* plants had the same signal as non-growing root hairs. Hairs in the root zone where there should be elongation have low signal (13.35±12.42 AU, *n*=89), not significantly different from mature root hairs in *glr3.7* (-5.64±18.17 AU, *n*=57), *ein2* or Col-0. This shows that GLR3.7 regulates root hair elongation by sustaining proper membrane depolarization at the tip, while EIN2 mediates other aspect of tip growth.

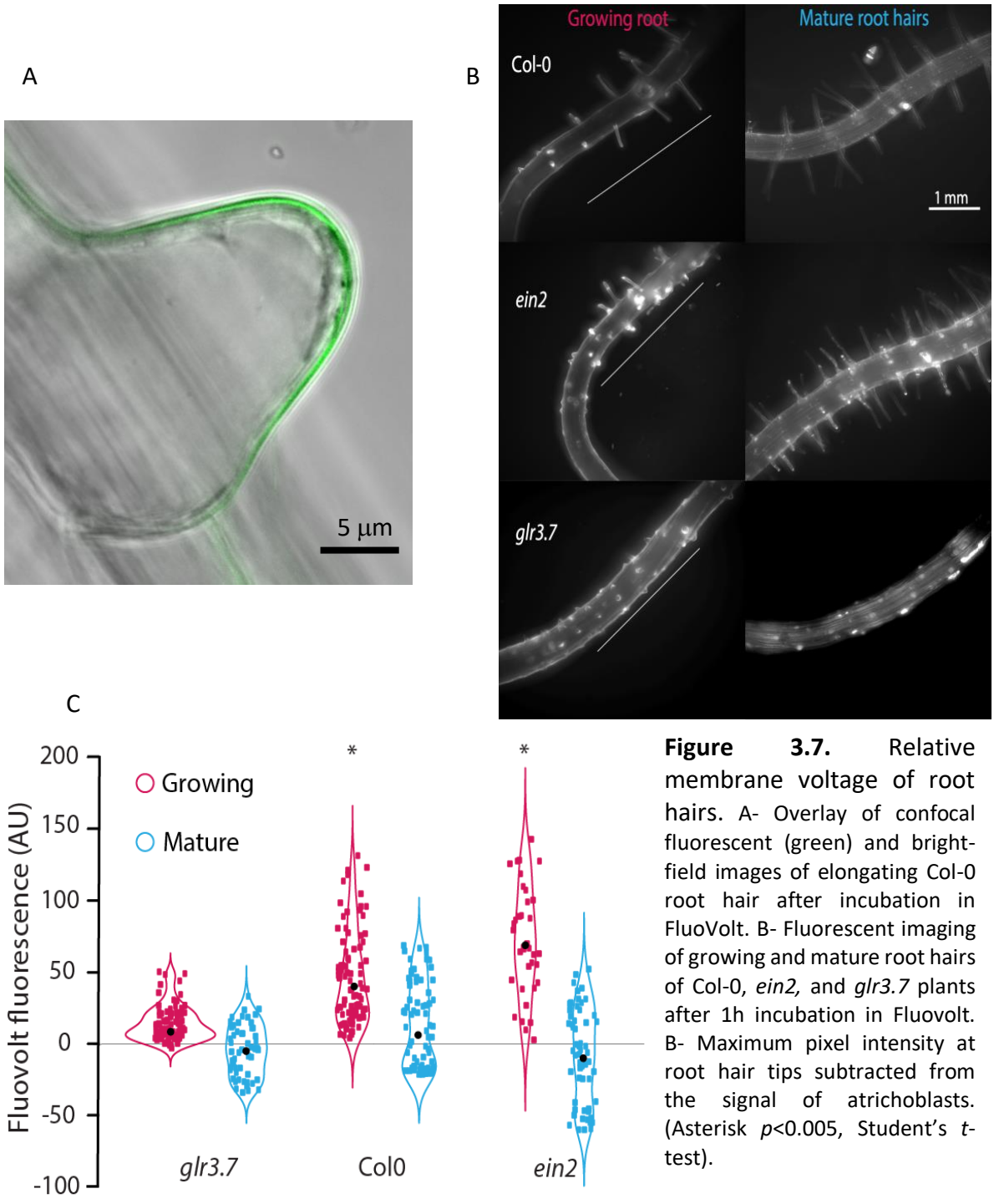



**Figure 3.7.** Relative membrane voltage of root hairs. A- Overlay of confocal fluorescent (green) and bright-field images of elongating Col-0 root hair after incubation in FluoVolt. B- Fluorescent imaging of growing and mature root hairs of Col-0, *ein2,* and *glr3.7* plants after 1h incubation in Fluovolt. B- Maximum pixel intensity at root hair tips subtracted from the signal of atrichoblasts. (Asterisk $p<0.005$, Student's *t*-test).

## Discussion

Despite the functional redundancy of GLRs, we were able to identify GLR3.7 as a receptor that leads elongation in *Arabidopsis* root hairs and sustains proper depolarization at the root hair tip. The elongation process is enhanced by exogenous amino acid application independently of the differentiation and initiation phases. As reported in literature, we confirm that the ethylene sensing pathway protein EIN2 also regulates root hair elongation and propose a model of interaction between GLR3.7 and EIN2 in this process.

*Root hair elongation is potentiated by amino acids*

Root hairs presented stronger elongation after application of proteinogenic amino acids, being the strongest response that to Glu. Given that the Glu solution is obtained by aqueous dilution of glutamic acid, the pH of the 1/2 MS solution containing 1mM Glu had to be carefully buffered to 5.7, given that it is the optimal pH for root hair elongation (Bibikova et al., 1998).

Furthermore, the presence of proteins in the growing medium promotes root hair elongation at a concentration of 5-20 µM, but inhibits it above 45 µM. This regulation is independent of the hormones auxin and ethylene

(Lonhienne et al., 2014). Also, 50μM Glu applied at the tip of a root is enough to arrest root growth (Walch-Liu et al., 2006), but the roots in the presented experimental setup, whether shorter, continue to grow and present root hair elongation, indicating that the effective concentration of Glu is significantly lower than the 1mM originally applied. It is necessary to experimentally determine the effective concentration required to induce this response, as the current experimental setup is insufficient in that regard.

The current research determines that the GLR mediated root hair elongation requires the activity of ethylene to mediate full root hair length. However, it does not approach the interaction of GLR3.7 with the auxin signaling pathway, a known regulator or root hair elongation. This is an important factor in the control network of this developmental process and should be investigated in further research.

*GLR3.7 drives root hair elongation through membrane voltage*

The root hairs of the mutant plant *glr3.7* initiate and bulge, a process potentiated by exogenous ethylene, similar to wild type. However, differentiated root hairs do not elongate even in the presence of amino acids

or ethylene, indicating that the activity of GLR3.7 occurs downstream of cell fate determination and the initiation cue of the RSL family proteins, auxin and ethylene.

The activity of GLR3.7 sustaining root hair elongation agrees with this channel´s role in pollen tube elongation (Michard et al., 2011). Likewise, GLR1.2 was also identified as a regulator of pollen tube elongation through the maintenance of $Ca^{2+}$ gradients in these elongating cells (Michard et al., 2011). It is likely that GLR3.7 also regulated root hair growth through the maintenance of the $Ca^{2+}$ gradient at the tip. This is supported by (1) the expression of GLR3.7 at the root hair tip membrane, (2) the disruption of root hair membrane voltage in *glr3.7* plants, (3) the increased elongation elicited by exogenous Glu, and (4) the $Ca^{2+}$-containing currents that are potentiated by Glu in heterologous cells expressing GLR3.7 (Simon, et.al., under review).

In and *glr3.1-2-3-6* knockouts, the response to amino acids is opposite to that of *glr3.7*: root hair length increases. This result is counter-intuitive and not easy to explain, except by recurring to generic explanations like functional redundancy of GLRs and possible overexpression of GLR3.7 in the absence

of other members of the family, or genetic compensation through other channels. Substantiating these hypotheses would require further investigation.

Root hair elongation is promoted by exogenous application of ACC, which could also activate GLR currents. It has been shown that GLR3.6, and GLR3.3 to a less extent, potentiate ionic currents of root cell protoplast in the presence of ACC (Mou et al., 2020). It is not known whether GLR3.7 can also be directly activated by ACC. But the exploration of how GLR3.7 affects those GLR3.3/6 ACC-triggered currents *in planta* might lead to physiological understanding of the gain of function by heteromerization of GLRs proposed by Simon et.al. (under review).

Furthermore, the considerably greater effect of ACC on elongation might be explained by its stronger potentiation than Glu of GLR mediated currents. Although possible, this does not explain the strongly decreased ACC-induced growth in *ein2* plants, which is suggestive of a cross talk between GLR and ethylene signaling.

*EIN2 is regulated by GLR3.7 action to potentiate root hair elongation*

Ethylene induces root hair elongation through EIN2 (Martín-Rejano et al., 2011; Pitts et al., 1998). Here we show that mutant *ein2* plants do present elongation, but it is diminished in comparison to the wildtype, indicating that EIN2 is not necessary for elongation but does enhance it.

Root hair elongation is associated with apical $Ca^{2+}$ oscillations (Candeo et al., 2017; Wymer et al., 1997), a process that occurs during elongation in a solution and in the air, which raised the hypothesis that $Ca^{2+}$ source can be extracellular from the surrounding solution, from the newly deposited cell wall, or internal from intracellular stores (Mendrinna and Persson, 2015). EIN2 is localized to the endoplasmic reticulum (Alonso et al., 1999), and the activity of the ethylene signaling pathway through EIN2 has been related to permeation of metals, and specifically $Ca^{2+}$ flux across internal membranes (Laohavisit and Davies, 2007; Petruzzelli et al., 2003). This $Ca^{2+}$ regulator could act in parallel with GLR3.7 and complement its activity to support the proper $Ca^{2+}$ oscillations that maintain root hair elongation.

However, the data here reported makes evident that GLR3.7 and EIN2 activities are not equally important. Amino acids induce root hair growth through GLR3.7 even in the absence of EIN2. On the contrary, without GLR3.7 there is absolutely no elongation, despite the presence of EIN2 (figure3.6-B).

Based on this evidence, we hypothesize that the complementation and crosstalk of the two parallel $Ca^{2+}$ regulators promote root hair elongation, a link that has been reported in wounded epidermal cells that present transient depolarization followed by ethylene increase (Marhavý et al., 2019). On one hand, amino acids signal membrane depolarization through GLR3.7-containing channels, which can be directly mediate by $Ca^{2+}$ influx through GLR3.7, or indirectly through anion-carried depolarization that activates voltage-dependent $Ca^{2+}$ channels. On the other hand, ethylene signaling, via EIN2, activates intracellular $Ca^{2+}$ movement that contributes to its proper homeostasis. This explains the stronger effect of ACC on root hair elongation, since it can boost the activity of the GLR pathway and the EIN2 pathway when converted to ethylene.

Finally, ethylene signaling is necessary for root hair elongation, but it is not sufficient; while GLR3.7 is both necessary and sufficient. The absence of elongation when EIN2 is present but GLR3.7 is not, suggests that the GLR signal is necessary for the EIN2 activity to occur. This putative coincidence detector could ensure the proper timing of root hair elongation because it integrates the ethylene signal existing from the differentiation and initiation stages, and a novel cue that triggers growth. Hence avoiding membrane deposition in an atrichoblast or a non-bulged trichoblast.

## Methods

### *Plant material*

*Arabidopsis thaliana* Col-0 and *glr3.7* (Salk_103942) were obtained from the Salk institute (La Jolla, CA, USA). *glr3.1-2-3-6* was developed and obtained from Edward Farmer (University of Lausanne, Switzerland). *Ein2* was obtained from Caren Chang (University of Maryland, USA). An *Arabidopsis* line was transformed with a GLR3.7::GFP linked construct under the expression of the endogenous promoter of GLR3.7.

Seeds were surface sterilized and sown on petri dished 0.8% agar with half strength Murashige-Skoog salt, and then stratified in 4º C for 24 hours. The

plates were then subjected to a light/dark regime of 16:8 at 20º C in a vertical position to allow roots to grow in a linear manner. 7-10 days old plants were used for data acquisition.

*Membrane potential measurements*

Membrane potential measurements were made by extracellular recordings in current-clamp mode. The root tip was first hydrated for 20 minutes with liquid 1/2MS (pH 5.7) prior to impalement. The dishes were placed on an inverted microscope and cells from the cortical cell layer, ≈15 μm deep into the tissue, were impaled at ≈2mm above the root tip. Only cells that presented a resting membrane potential below -120mV were used for stimulation. The impalement was done with a sharp electrode, resistance >100MΩ, filled with 3M KCl. Pipettes were pulled using a P97 puller (Sutter Instrument, Novato, CA, USA). The membrane voltage was tracked at a sampling frequency of 2-4 kHz and filtered at 1-2 kHz using an Axopatch 200A amplifier with an Axon 1200 DigiData analog-to-digital converter and pCLAMP software (Molecular Devices, Sunnyvale, CA, USA).

The tissue stimulus was applied with a 1μl drop delivered by a 1mm thick capillary placed at the tip of the root and controlled by a CellTram microinjector (Eppendorf AG, Hamburg, Germany). Treatments of D-

mannitol and L-Glu (Sigma-Aldrich, St. Louis, MO, USA) were applied at 1mM on liquid 1/2MS and balanced to pH5.7 with BTP.

### *Amino acid stimulation*

Roots of seedlings between 7 – 10 days old were stimulated with 5µl of D-Serine, L-Cysteine, L-Asparagine, 1-Aminocyclopropano-1-carboxylic acid (Sigma-Aldrich, St. Louis, MO, USA), L-Glutamic Acid, L-Serine, L-Methionine (Fisher Scientific, Hampton, NH, USA), balanced to pH5.7, and diluted to 1mM or 0.1mM in half strength Murashige-Skoog salt pH 5.7.

### *Root hair imaging*

Observation of roots was done using an MVX-ZB10 Olympus stereozoom (Olympus America Inc., MA, USA). Bright field illumination was provided by a Schott KL300 LED white light casted parallel to the root growth. Image acquisition was done with a Hamamatsu Flash4.0 V3 sCMOS digital camera at a magnification of 15x. Confocal images were obtained using a Zeiss LSM 980 confocal Airyscan2.

*Membrane voltage imaging*

Roots of seedlings between 7-10 days old were imbibed in 200µl half strength Murashige-Skoog salts pH5.7 with 4µl Power Load and 0.5µl FluoVolt (Waltham, MA, USA) during 1 hour prior to imaging.

Fluorescence observation was done in an MVX-ZB10 Olympus stereozoom (Olympus America Inc., MA, USA). Fluorescent illumination was provided by a MVX coaxial fluorescent illuminator with a GFP filter set with excitation: 470/40 nm, dichroic: 495 nm long-pass, emission: 525/50 nm. Image acquisition was done with a Hamamatsu Flash4.0 V3 sCMOS digital camera at a magnification of 126x.

*Image analysis*

Root hair length, quantity and pixel intensity was determined using FIJI. Pixel intensity was determined as the average of the 10 pixels surrounding the maximum value of a cross section at the tip of the root hair where the dome meet the cylinder that conforms the hair. All data was manipulated and statistically tested in R Core Team (2020) (R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) and SigmaPlot (Systat, San Jose, CA, USA).

# General conclusions

The data herein presented postulates GLR3.7 as an important mediator of root hair apical growth and amino acid-triggered electric signaling. The capacity of membrane depolarization is a mechanism common to both phenotypes, and in both cases is impaired in *glr3.7* mutants.

In the case of electric signaling, the glr3.7 mutant presented a response of diminished amplitude when stimulated with Glu (1mM). In this case, we hypothesize that the absence of GLR3.7 in GLR heteromers impairs the proper function of the indispensable channels GLR3.3 and GLR3.6. The diminished conductivity or gating properties would be observed as a diminished cellular response to the ligand stimulus.

Along with this, we demonstrated that the depolarization and cytosolic $Ca^{2+}$ increase that result from ligand stimulation do not propagate from cell to cell and share kinetics with the initial phase of the SWP. This allows us to propose that the chemical propagation theory of SWP accounts only for the initial depolarization and for the $Ca^{2+}$ transient. Also, another stimulus, likely related to the propagation of a hydraulic wave, that complements the ligand-triggered response, and sustains the SWP depolarization for longer is yet to be proposed.

The importance of GLRs in ligand-induced depolarization and $Ca^{2+}$ transient, and the lack of understanding on the ionic species that underlie these signals, makes evident the necessity of understanding the selectivity of the GLRs involved in SWPs. Specifically, GLR3.3/6, and how the interaction with GLR3.7 regulates the selectivity and gating properties. This is essential to direct future research to determine whether the GLR-mediated depolarization is underpinned by $Ca^{2+}$ influx or by anionic efflux, from which further interaction with other channels can be investigated.

Also, to understand the interactions network that produces the SWP, the field is in debt with the genetic identification of the channels implicated with other parts of the signal i.e., voltage dependent anion or $Ca^{2+}$ channels in the depolarization, and voltage dependent $K^+$ and $H^+$ channels in the repolarization phase, as well as identification of mechanosensitive channels accountable for the transduction of the hydraulic change in SWP propagation.

Similar to the ligand-induced electric response, we demonstrated that the presence of GLR3.7 is necessary for proper membrane depolarization in root

hair elongation. The root hairs of the *glr3.7* mutant did not elongate and also did not present apical depolarization, as did the root hairs of the Col-0 plants.

Moreover, application of Glu promotes longer root hairs in wildtype but not in *glr3.7* plants. It is possible that this stimulus had non-desired effects on root hair development through pH unbalance. Although the pH was carefully buffered, it is important in the future to quantify the pH in root hairs upon Glu stimulation, which could be done with fluorescent pH sensors. Glu can affect the development of roots through the unbalance of protein status

In order to dissect the regulation of root hair elongation by Glu through membrane receptors from that of hormones through metabolic processes, this work studied the interaction between GLRs and the ethylene pathway. However, it is necessary to direct future research towards dissecting these pathways from auxin signaling.

GLR3.7 sustains apical membrane depolarization of elongating root hairs. Nevertheless, it is yet unknown how this affects the $Ca^{2+}$ gradient in tip elongation. It is possible that GLR3.7 is permeable to $Ca^{2+}$ and directly sustains this gradient and depolarization, or it could be sustaining an anionic-

sustained depolarization to regulate voltage sensitive $Ca^{2+}$ channels. Identification of the GLR3.7 ionic selectivity in root hairs is necessary and will allow the field to understand the feedback loops between membrane potential, cytosolic $Ca^{2+}$ and other membrane channels that sustain the oscillating nature of this gradient.

Altogether, this work postulates GLR3.7 as a contributor to membrane voltage regulation, a process common to chemical signaling and cellular tip growth. Further research is necessary to determine the pore conductance for which GLR3.7 is responsible in each specific case. This will allow the integration of GLR3.7 in the network of channels that support cell signaling and development.

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