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“*AtGLR3.7* as negative regulator of *AtGLR3.3* mediated stress responses”

STEFANO BURATTI

TUTOR: Prof. Alex Costa

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1. Abstract

During evolution, plants have developed sophisticated mechanisms to perceive and respond to environmental cues. Adaptability and responsivity to biotic and abiotic stresses involve a complex interplay of multiple factors: second messengers, phytohormones, metabolites and a whole variety of molecules involved into the perception, distribution, and transduction of stress signals. Recent studies have demonstrated that wounding-driven long-distance propagation of Ca^{2+} waves depend on the activity of the cation permeable channel Glutamate Receptor-Like 3.3 (GLR3.3), but the mechanisms regulating this crucial factor remain largely unknown.

GLRs are homologous to the animals' ionotropic glutamate receptor channels (iGluRs), which can form both homo and hetero-tetramer. This allows us to hypothesize that different assemblies of plant GLRs as well can bring to the formation of channels with different properties.

Here we present multiple lines of evidence supporting the molecular and functional interdependency between GLR3.3 and another relatively uncharacterized GLR isoform, GLR3.7.

Preliminary data in our laboratory indicate that GLR3.7 activity can negatively impact GLR3.3-mediated responses, such as burning and exogenous amino acid administration, suggesting this GLR isoform as a putative partner of GLR3.3.

Colocalization and close proximity of GLR3.3 and GLR3.7, both at tissue and cellular level, has been demonstrated by GUS reporter assay, complemented by a FLIM analysis. Moreover, Co-Immunoprecipitation experiments demonstrated direct protein-protein interaction between the two GLRs.

Intriguingly, expression of the cation permeable *GLR3.3* channel exhibits a negative impact on yeast G19 sodium sensitive strain growth efficiency, significantly rescued by *GLR3.7* expression, indicating a wider influence of GLR3.7 on GLR3.3 activity.

To investigate the relationship between GLR3.3 and GLR3.7 activity in response to changes of natural environmental conditions, we developed a new protocol to study the effect of re-watering drought-stressed plants. In this scenario, GLR3.3 showed again a crucial role in the generation and propagation of long-distance Ca^{2+} waves, whereas GLR3.7 exerts a negative regulatory role.

In summary, our results suggest that GLR3.7 can modulate the activity of GLR3.3 by possibly forming, *in planta*, a heteromeric channel.

2. Introduction

2.1. Ca^{2+} at the crossroads between biology and chemistry

Key among all living organisms, calcium ions (Ca^{2+}) serve as potent messengers, orchestrating a delicate balance between their critical role in biological processes and their potentially harmful cytotoxicity. Within the intricate environment of a cell, Ca^{2+} affects the stability of lipid membranes, nucleic acids, and proteins, necessitating strict regulatory mechanisms to maintain cellular homeostasis. Plants are not an exception: in the cytosol, free calcium ($[\text{Ca}^{2+}]_{\text{cyt}}$) is maintained in the nanomolar range (50-100 nM) (Logan & Knight, 2003; Stael et al., 2012). From the most ancient bacteria to the most evolved multicellular organisms the only effective strategy for survival has been to forcibly export Ca^{2+} from this delicate subcellular compartment (Case et al., 2007).

The evolution of Ca^{2+} -permeable channels, a key component of this intricate regulation, reflects the pervasiveness of Ca^{2+} gradients across diverse life forms: in fact, Ca^{2+} -permeable channels are phylogenetically among the oldest and present in many ancient prokaryotes (Shemarova & Nesterov, 2005). These complex transmembrane proteins feature a challenge, the steep Ca^{2+} gradient is a powerful electrochemical force and to work against such a force is not an easy task. Channels often pair with a strong motive energy to force Ca^{2+} ions against their own electrochemical gradient, either through the hydrolysis of ATP or proton motive force (Demidchik et al., 2018).

In biology nothing is wasted, the consumption of these reserves of energy also creates across membranes the steep Ca^{2+} electrochemical gradients cited above, which are promptly exerted by the cell as a fast and responsive signaling system: extracellular perturbations can be transduced to the intracellular space in the form of a rapid influx of Ca^{2+} ions, promptly perceived by the cell (Dodd et al., 2010).

In plants, Ca^{2+} is a ubiquitous second messenger involved in the perception and response to a wide range of stimuli (Grenzi, Bonza, et al., 2021; Kudla et al., 2018; Monshausen, 2012; Resentini, Ruberti, et al., 2021; Sanders et al., 2002). These stimuli induce a specific $[\text{Ca}^{2+}]_{\text{cyt}}$ increase, and plants are able to discern these stimuli from the others. This precision is possible thanks to either the fine regulation of Ca^{2+} movement through lipid bilayers, and the existence of a battery of Ca^{2+} sensor proteins (Kudla et al., 2018). The regulation of a precise and highly regulated Ca^{2+} movement across membranes translates into stimulus-specific " Ca^{2+} signatures" characterized by unique patterns of amplitude, frequency, and duration of $[\text{Ca}^{2+}]_{\text{cyt}}$ increases (McAinsh & Hetherington, 1998).

These signatures are swiftly perceived by the plant cell through the activation of different Ca^{2+} sensors (e.g. Calmodulin (CaM), CaM-Like proteins, Calcineurin b-like proteins) or calcium relays (Ca^{2+} -dependent protein kinase or CaM-dependent protein kinase). These signaling cascades orchestrate the cellular responses, regulating gene expression and metabolism, ensuring plant survival and adaptability in the face of environmental perturbations (Kudla et al., 2018; Whalley et al., 2011).

2.2. Ca^{2+} homeostasis: Ca^{2+} importers and exporters

In plants, perception of external signals may lead to rapid elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$, an event resulting from the passive Ca^{2+} influx mainly through Ca^{2+} -permeable channels localized in the plasma membrane and/or in the membranes of subcellular compartments (Kudla et al., 2018) but also by the inactivation or downregulation of active Ca^{2+} efflux systems (pumps and transporters). In plants, at least four different families of Ca^{2+} -permeable channels do exist: Cyclic Nucleotide Gated Channels (GNGCs), Mechanosensitive Channels (MCAs), reduced hyperosmolarity-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase (OSCs), and Glutamate Receptor-Like channels (GLRs) (Kudla et al., 2018). As stated above, a high $[\text{Ca}^{2+}]_{\text{cyt}}$ is not compatible with life, so the activity of Ca^{2+} -permeable channels must be tightly regulated (Yan et al., 2024).

At the beginning of this introduction, we reported that Ca^{2+} is highly reactive with biological molecules. In fact, a pool of Ca^{2+} binding proteins (nucleic acids phosphates, anionic lipids heads, carboxyl oxygen groups in proteins) buffer partially the excess of Ca^{2+} (Demidchik et al., 2018), but far from being enough to singlehandedly contain such a burst of free cation.

2.3. Plant Ca^{2+} ATPases: From biochemistry to signalling

The full removal of Ca^{2+} from the cytosol is a demanding process: it is an energy-dependent activity mediated by $\text{Ca}^{2+}/\text{H}^{+}$ antiporters, powered by the proton-motive force, P1-ATPases and Ca^{2+} -ATPases, powered by ATP hydrolysis. Of the latter we can find two groups in plants, the IIA subgroup ECAs (ER-type Ca^{2+} -ATPase), orthologs of animal SERCA, and the IIB subgroup ACAs (Auto-inhibited Ca^{2+} -ATPase), related to animal PMCA (Costa et al., 2023).

Together these systems allow for a fine and precise tuning of Ca^{2+} homeostasis in the whole cell.

In this review, we provide an overview on the regulation and roles in Ca^{2+} signalling of the type II Ca^{2+} -ATPases *in planta*.

Type II Ca^{2+} -ATPases feature multiple homologues, populating different tissues and subcellular compartments, and complex regulatory mechanisms: Type IIB Ca^{2+} -ATPases are mainly localised at the plasma membrane and at the endomembranes, including endoplasmic reticulum (ER) and tonoplast, and their activity is regulated by a CaM - Ca^{2+} -dependent mechanism, which removes the inhibition of the N-terminal autoinhibitory domain. Type IIA ER-type Ca^{2+} -ATPases (ECAs) on the other hand are present mainly at the ER and Golgi Apparatus membranes and are active at resting Ca^{2+} levels. The presence of multiple homologue genes in plant genomes suggest that slight differences in subcellular localization, tissutal specificity or regulative properties, contributes to specific Ca^{2+} signalling during plant growth and development (this part has been extensively reviewed in Costa, Resentini, Buratti & Bonza, 2023).

2.4. Calcium sources

Free $[\text{Ca}^{2+}]_{\text{cyt}}$ normally ranges from high nanomolar to low micromolar concentrations, while subcellular compartments can vary widely. The cell wall exhibits low millimolar concentrations of free Ca^{2+} (S. J. Conn et al., 2011), while vacuoles span a wide range from 0.2 to 50 mM (S. Conn & Gilliam, 2010). Chloroplast stroma harbours a free Ca^{2+} concentration of around 100 nM (Loro et al., 2016; Sello et al., 2016; Stael et al., 2012), the mitochondria matrix maintains a resting value of about 200 nM (Logan & Knight, 2003; Loro et al., 2012; Stael et al., 2012), and the lumen of the endoplasmic reticulum (ER) is estimated to contain 50–500 μM of free Ca^{2+} (Bonza et al., 2013; Iwano et al., 2009; Resentini, Grenzi, et al., 2021; Resentini, Ruberti, et al., 2021).

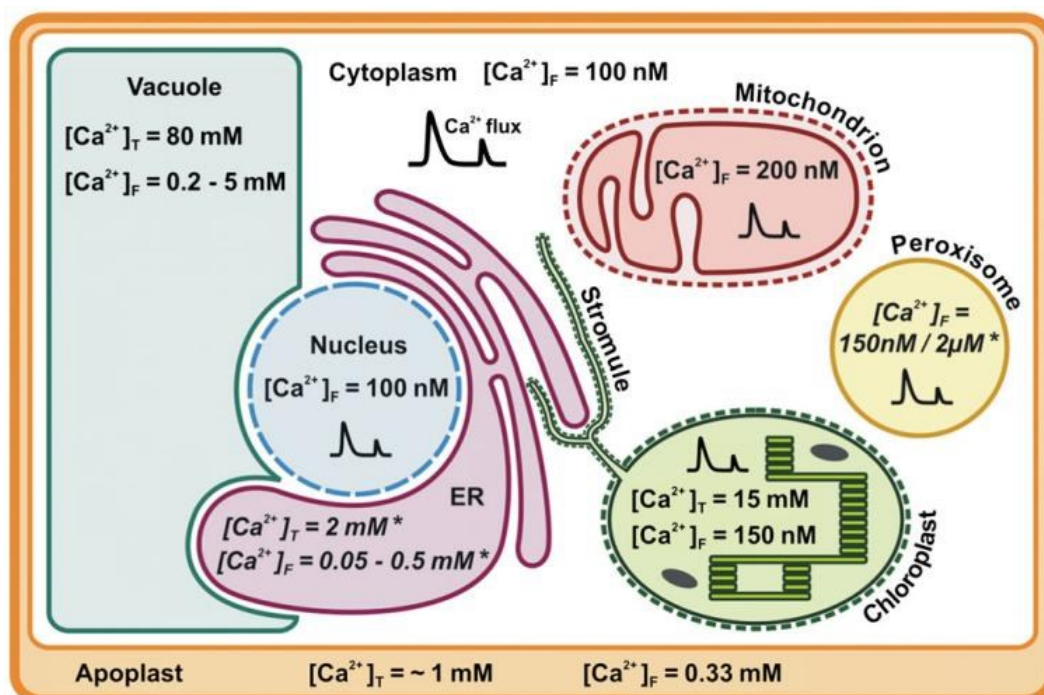


*Figure 1 - Summary of organellar Ca^{2+} concentrations in the plant cell. Approximated reported values for reported total ($[\text{Ca}^{2+}]_T$) and free resting ($[\text{Ca}^{2+}]_F$) Ca^{2+} concentrations in the different organelles (apoplast, cytoplasm, vacuole, nucleus, ER, chloroplast, mitochondrion, and peroxisome). *The given values are taken from the animal field due to limited information in plants. Calcium fluxes are illustrated by a double peak-shaped symbol. Extracted from Stael et al., 2012*

While most literature reports the apoplast as the main source of free Ca^{2+} , the involvement of organelles in shaping the Ca^{2+} signature is undeniable. As an example, Ca^{2+} -ATPases involved in $[\text{Ca}^{2+}]_{\text{cyt}}$ homeostasis can be found in multiple subcellular membranes, like the nuclear envelope, the ER, the Golgi apparatus, the tonoplast and the plasmalemma (Costa et al., 2023). The nucleus, and chloroplasts are even able to generate their own Ca^{2+} signatures (reviewed in Resentini, Ruberti, et al., 2021; Stael et al., 2012) and the ER is even reported as a Ca^{2+} capacitor that after $[\text{Ca}^{2+}]_{\text{cyt}}$ can remove excess Ca^{2+} from the cytosol (Bonza et al., 2013; Resentini, Grenzi, et al., 2021; Resentini, Ruberti, et al., 2021).

Given the intricate interplay of mechanisms involved in Ca^{2+} homeostasis, it is evident that the generation of " Ca^{2+} signatures" is not a straightforward process. Instead, it develops from a delicate synergy between the perception of extracellular signals, intracellular responses, and finely tuned mechanisms within the cell.

2.5. Evolutionary origin of Glutamate Receptor-like channels (GLRs)

Among the different gene families coding for Ca^{2+} -permeable channels, the GLR family has twenty different isoforms in *Arabidopsis thaliana* (henceforth Arabidopsis) (Wudick, Michard, et al., 2018). In Arabidopsis, the GLR family is grouped into three different clades, and its members are involved in several physiological roles: like fertility, root development, stomatal aperture, and biotic and abiotic stress responses (Grenzi, Bonza, et al., 2021; Wudick, Michard, et al., 2018).

Plants GLRs are homologues of Ionotropic Glutamate Receptors Channels (iGluRs) that are ligand-gated ion channels that mediate most of the fast synaptic transmission in the nervous system (Hansen et al., 2021; Traynelis et al., 2010). The structure of iGluRs is heavily conserved, with an extracellular amino-terminal domain (ATD), followed by the ligand-binding domain (LBD), three full transmembrane domains (TMD), one partial TMD and a cytosolic carboxyl-terminal domain (CTD). To properly function as ion permeable channels, the iGluRs tetramerize, forming a functional pore. The binding of the agonist induces a conformational change in the LBD, transmitted to the transmembrane helices, causing the opening of the channel pore (Traynelis et al., 2010). iGluRs can exist as both homo-tetrameric and hetero-tetrameric channels, which gives rise to a wide range of plasticity in terms of channel activity (Traynelis et al., 2010). iGluRs can be found in all neuronal

cells, and they mediate the post-synaptic neuron influx of cations after the pre-synaptic button release of neurotransmitters (e.g. L-Glutamate, Glycine and D-Serine), allowing excitatory signal propagation (Traynelis et al., 2010).

Evolutionary speaking, plants GLRs have parallelly evolved to iGluRs (Wudick, Michard, et al., 2018). They are all predicted to come from bacteria, in particular the primary sequence of these glutamate receptors highly resembles bacterial proteins: iGluRs LBD resembles bacterial glutamine binding proteins (M. Price et al., 2012), while the main organization of their transmembrane portion widely overlaps with inverted tetrameric potassium channels like KcsA (MacKinnon, 2003), so possibly iGluRs come from a fusion event after speciation. GLRs do resemble iGluRs structure, featuring the three full and one partial transmembrane domain (aminoacids identity similarity between 16-63% among TMDs (Chiu et al., 1999) and the LBD, and while it was predicted that GLRs did not possess an ATD, important for iGluRs tetramerization and modulation (Jin et al., 2009; Lipton et al., 1997), recent Cryo-EM structural analysis of *At*GLR3.4 proved otherwise (Green et al., 2021). Phylogenetic analysis did not show any closer association between plants and metazoan or bacteria GLRs homologs, indicating a very ancient and simultaneous separation of the three (M. Price et al., 2012).

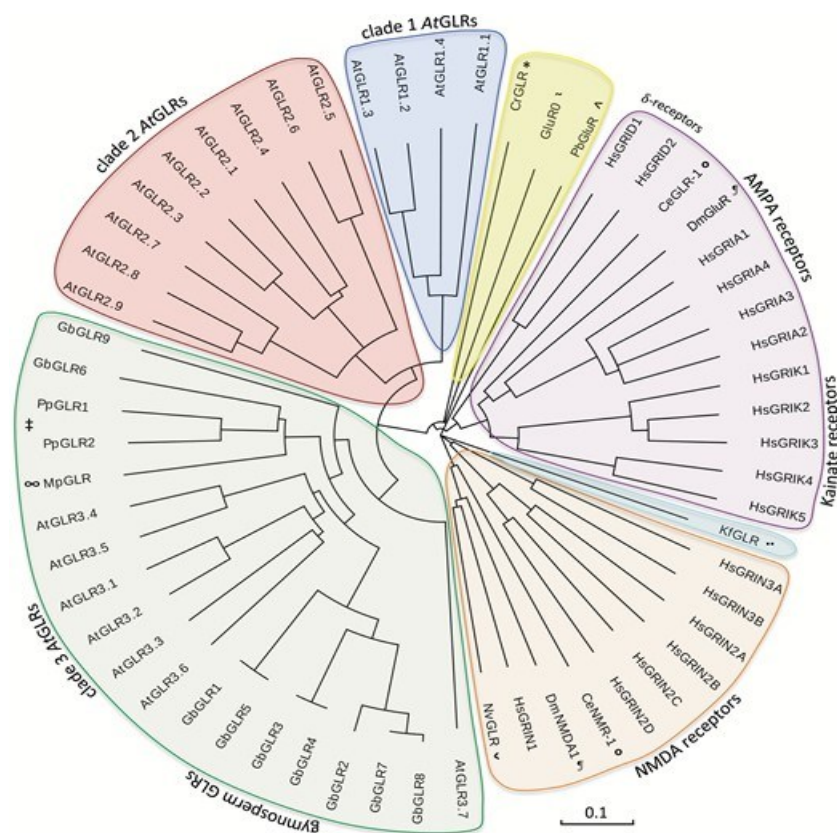


Figure 2 - Phylogram glutamate receptors from bacteria, plants, and animals. The tree shows the phylogenetic relationship of chosen GLRs from *Arabidopsis thaliana* (At), *Caenorhabditis elegans* (Ce, °), *Chlamydomonas reinhardtii* (Cr, *), *Drosophila melanogaster*

(*Dm*, ¶), *Ginkgo biloba* (*Gb*), *Homo sapiens* (*Hs*), *Klebsormidium flaccidum* (*Kf*, ~), *Marchantia polymorpha* (*Mp*, ∞), *Nematostella vectensis* (*Nv*, ~), *Physcomitrella patens* (*Pp*, ‡), *Pleurobrachia bachei* (*Pb*, ^), and *Synechocystis* sp. (*GluR0*, ~). The scale bar indicates substitutions per site. From Wudick, Michard, et al., 2018

After this first branching, GLRs steadily evolved, thanks to duplication events, from only two predicted homologs in the Streptophyta *Marchantia polymorpha*, to the predicted sixteen homologs in the monocotyledon *Oryza sativa* and thirteen in the dicotyledon *Solanum lycopersicon* (De Bortoli et al., 2016), and twenty isoforms in *Arabidopsis* (Wudick, Michard, et al., 2018), our model organism. The genes resulting from these duplications events have been divided into four different clades based on sequence homology, Clade III, more ancient, Clade I and II considered as sister clades, and Clade IV, less diffused and absent in brassicales, like our model organism *Arabidopsis* (J. Chen et al., 2016).

GLRs participate in the plant response to external stimuli and the wide diffusion of GLRs is a clear hint at their importance. As mentioned earlier, GLRs homologs in animals are key players in nervous system activity and long-distance propagation of neuronal excitation. Plants do not feature any kind of organ resembling nervous system organization or activity, so how do they transmit long-distance signals?

Even if devoid of a nervous system, electric signaling has long been associated with plant perception of the surrounding environment. In 1873, the first evidence of an Action Potential (AP) in plants was documented in *Dionaea muscipula* (Burdon-Sanderson, 1873). Microelectrodes recorded *Nitella* cells APs even earlier than their first registration in animals (Umrath, 1930), and similar AP-like electrical signals were recorded in *Mimosa pudica* (Samejima & Sibaoka, 1982)(Samejima & Sibaoka, 1982). After only a couple of years, evidence of ion channels activity participating in APs were discovered in plants (Schroeder et al., 1984). Remarkably, scientists found common mechanisms for generating and regulating electric signals in plants and animals, sharing key elicitors and cellular messengers between the two kingdoms (Fromm & Lautner, 2007).

Plants developed their own sophisticated electric signaling system. This system uses the specialized tissues of vascular bundles to rapidly transmit perturbations throughout the plant over long distances, triggering tailored responses to environmental challenges. As an example, in 2013, it was demonstrated that local leaf wounding in *Arabidopsis*, not only triggers local and distal propagation of electrical signal, but also the accumulation of Jasmonic Acid (JA) and expression of JA-responsive genes in systemic leaves sharing vascular connections with the wounded one (Mousavi et al., 2013). This breakthrough was particularly interesting also because at the base of this mechanisms laid plants iGluRs homologs, GLR3.3 and GLR3.6.

2.6. Long-distance stress signaling and GLRs

As discussed earlier, Mousavi and colleagues demonstrated that leaf wounding triggers long-distance electrical signals, that are fundamental for the subsequent accumulation of JA in systemic tissues. Importantly, both events depend on the GLRs activity. Knocking out of two GLRs from clade III, GLR3.3 and GLR3.6, was sufficient to eliminate long distance electric signals and substantially reduce JA-responsive genes upregulation.

This result suggests that GLRs, similarly to animal iGluRs, are part of the machinery necessary for long distance propagation of electric signals and the resulting molecular response. Even their localization supports this hypothesis: GLR3.3 and GLR3.6 were localized in the phloem and xylem parenchyma cells, respectively, suggesting that these two vascular tissues are, theoretically, able to sustain the propagation of electrical signals. For example, sieve tubes (one of the cell types belonging to phloem), as their name suggest, are tubes originated from degenerated cylindrical cells that share their cytoplasm in a structure supported by the activity of companion cells (Haritatos et al., 2000). It may seem a similar structure to an axon, but it's important to note that the physical limitations of the phloem itself could make generating and modulating long-distance electric signals challenging: APs rely on passive diffusion of charges, limited by the presence of the non-conductive sieve plates at regular intervals along the phloematic tube. Companion cells, on the other hand, do not provide an insulation of the sieve tube, such as myelin does for axons (Hedrich et al., 2016). Also, while the phloem could function as conductive tissue for electrical signals, surface electrodes, that are usually employed to measure electrical signals propagation, can easily record such signals, implying that other cell types could dissipate the long-distance propagation of electric pulses (Farmer et al., 2014).

Taken together, this piece of information seems to oppose to a model in which electric signaling, dependent on GLRs, could propagate distally through phloematic tissues. If so, what is mediating such a fast and capillary diffusion of stress responses?

In 2014, Farmer and colleagues proposed a novel model, dubbed "Squeeze cell hypothesis", which suggests that electrical signaling could be coupled to an even faster physical force, hydrostatic pressure (Farmer et al., 2014), through a different main "actor", the xylem. The xylem is a tissue where its vascular vessels are real tubes, originated from total degeneration of cells, leaving behind an empty and continuous vessel that directly connects the root vascular bundle with leaves and flowers. These vessels are under a constant negative pressure, essential for the movement of water against gravity (Lazar & E. Zeiger, 2003). Local damage could induce a change in hydraulic pressure

that propagates along the xylem vessels to distal tissues, where this pressure change could be transferred to the xylem contact cells, a population already hydrostatically coupled to the xylem vessels. Electrical signals, dubbed “slow wave potentials” (SWPs) (reviewed in Fromm & Lautner, 2007) are not considered the main driving force of long-distance signal propagation, but a consequence. The radial pressure emanating from the xylem vessel could impose a physical pressure on neighbouring cells activating mechanosensors on the membrane. Aside from mechanosensors this could also cause the activation of other ion channels, including GLRs, regulating ion influx and SWPs generation. GLRs possess the potential to generate SWPs. Pore domains of GLR1.1 and GLR1.4 are permeable to Na^+ , K^+ and Ca^{2+} ions upon L-Glutamate treatment (Tapken & Hollmann, 2008), GLR3.4 generates cationic currents partially dependent on Ca^{2+} (Vincill et al., 2012; Green et al., 2021), GLR3.2 and GLR3.3 were also shown to be permeable to cations, and inhibited by Gadolinium (Gd^{2+}), an inhibitor cation-permeable channels (De Vriese et al., 2018).

In 2023, Bellandi and colleagues proved that wounding one cotyledon triggers a bulk flow within the vasculature, reaching speeds comparable to long-distance Ca^{2+} waves. They also demonstrated that the progression of the Ca^{2+} wave closely follows the leading edge of an apoplastic L-Glutamate $[\text{Glut}]_{\text{apo}}$ wave propagating from the site of stress application. Accordingly, to their interpretation, wounding causes a vascular damage able to induce a bulk flow carrying various molecules from the wounded site to the whole plant. Among this molecules, amino acids (AAs) readily activate GLRs, particularly GLR3.3 (Alfieri et al., 2020), initiating a subsequent Ca^{2+} wave.

2.7. Long-distance turgor pressure changes induce local activation of plant glutamate receptor-like channels

In 2023, we provided evidence that hydrostatic pressure, generated from leaf mechanical damage or osmotic stress applied to roots, is possibly the driving force of long-distance stress propagation.

We observed that a stress, like burning one of the Arabidopsis rosette leaves, resulted in a movement of the main inflorescence stem of Arabidopsis plants. Notably, this event was always preceded by an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in WT plants, but even in glrs mutant background devoid of this Ca^{2+} elevation, the movement persisted (Grenzi et al., 2023).

The bending of the stem can be possibly ascribed to the generation of a pressure wave induced by the leaf burning and propagating from the injured site to distal tissues, followed by an elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$. Our model takes into consideration the possibility that the pressure wave can induce the accumulation of glutamate in the apoplast, in turn activating the GLR3.3 localised in the phloem

and its gating is then necessary for the cytosolic Ca^{2+} increase. Indeed, in Grenzi et al. (2013) we showed that exogenous administration of glutamate induces a GLR3.3-dependent $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in the phloem. This result is also supported by Bellandi and colleagues, who showed that the Ca^{2+} signal propagation front, in laser-wounded cotyledons, lags behind apoplastic glutamate accumulation. In summary, we propose that after the application of a stress, a pressure wave occurs and, in systemic tissues, this triggers an apoplastic glutamate concentration ($[\text{Glu}]_{\text{apo}}$) increase, closely followed by $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation.

This signal propagation results into a subsequent plant stress adaptation, as demonstrated by the elevation of expression levels in distal tissues of the plant of the Jasmonate responsive gene JAZ10, as well as the zinc-finger protein (ZAT12), another reliable marker gene for multiple stress conditions like cold and oxidative stress (Davletova et al., 2005).

We want to highlight that, while the Ca^{2+} wave, and subsequent genetic response, was solely mediated by GLR3.3 activity, the $[\text{Glu}]_{\text{apo}}$ wave was largely independent. Additionally, we were able to show that the sole imposition of a local osmotic stress can generate responses like wounding and burning. As already documented in our lab, while hyperosmotic stress on root tips shows only a shrinkage of the tissue, the hypoosmotic stress caused a clear cytosolic Ca^{2+} increase linked to a swelling of the root back to its original size, which was again strongly dependent on GLR3.3 (Corso et al., 2018).

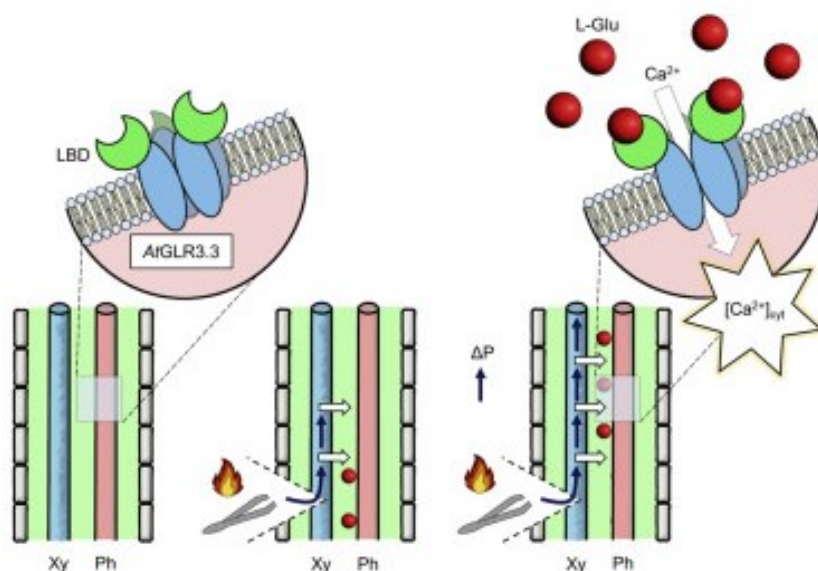


Figure 3 – Graphical representation of long distance propagation of local mechanical damage in Arabidopsis. Leaf mechanical injury, such as wounds and burns, as well as hypo-osmotic stress in root cells, induces the systemic apoplastic increase of L-Glu), which is largely independent of AtGLR3.3 that is instead required for systemic cytosolic Ca^{2+} elevation. Xy, Xylem vessel; Ph, Phloem vessel. From Grenzi et al., 2023

Our model predicts that the long-distance signaling in response to wounding, burning and osmotic stress is mediated by alterations in the vascular turgor pressure and the consequent water flux (Fig. 3) (Grenzi et al., 2023). This in turn, facilitates the movement of various molecules, including AAs like L-Glutamate, which are accumulated in the apoplast leading to the activation of GLR3.3, required to generate the subsequent SWPs and $[Ca^{2+}]_{cyt}$ increase. Later, the molecular cascade orchestrated by $[Ca^{2+}]_{cyt}$ accumulation induces plant adaptation to the stress through the regulation of gene expression.

This result suggested us that investigating the regulatory pathways of GLR3.3 activity could provide novel insights on stress perception and propagation.

2.8. GLR3.3 regulation

The crucial role of GLR3.3 as a key player in long-distance stress signaling has been firmly established, but only recently we started to better understand its regulation and biochemistry (Alfieri et al., 2020; Grenzi et al., 2023; Wudick, Michard, et al., 2018; Yan et al., 2024). Until recently, scant information existed regarding the gating and regulation of GLRs.. GLRs were initially thought to be gated primarily by L-Glutamate and a few related AA, like their animal iGluR homologs. However, our research has demonstrated that the ligand-binding domain (LBD) of GLR3.3 binds a variety of AA, that aside from L-Glutamate include L-Cysteine, L-Methionine, L-Alanine, L-Asparagine, L-Serine, and Glycine, with affinities in the low micromolar range (Alfieri et al., 2020). We have found that the AA efficacy, defined as the maximum $[Ca^{2+}]_{cyt}$ increases measured in Arabidopsis root cells, is proportional to their *in vitro* affinity for the GLR3.3 LBD (Alfieri et al., 2020).

This “ligand promiscuity” has also been predicted, and in some cases experimentally proved, for other isoforms like GLR1.2, GLR1.4, GLR3.2, GLR3.4 and GLR3.1/GLR3.5 (Gangwar et al., 2020, 2021; Green et al., 2021; Kong et al., 2016; Michard et al., 2011; Tapken et al., 2013).

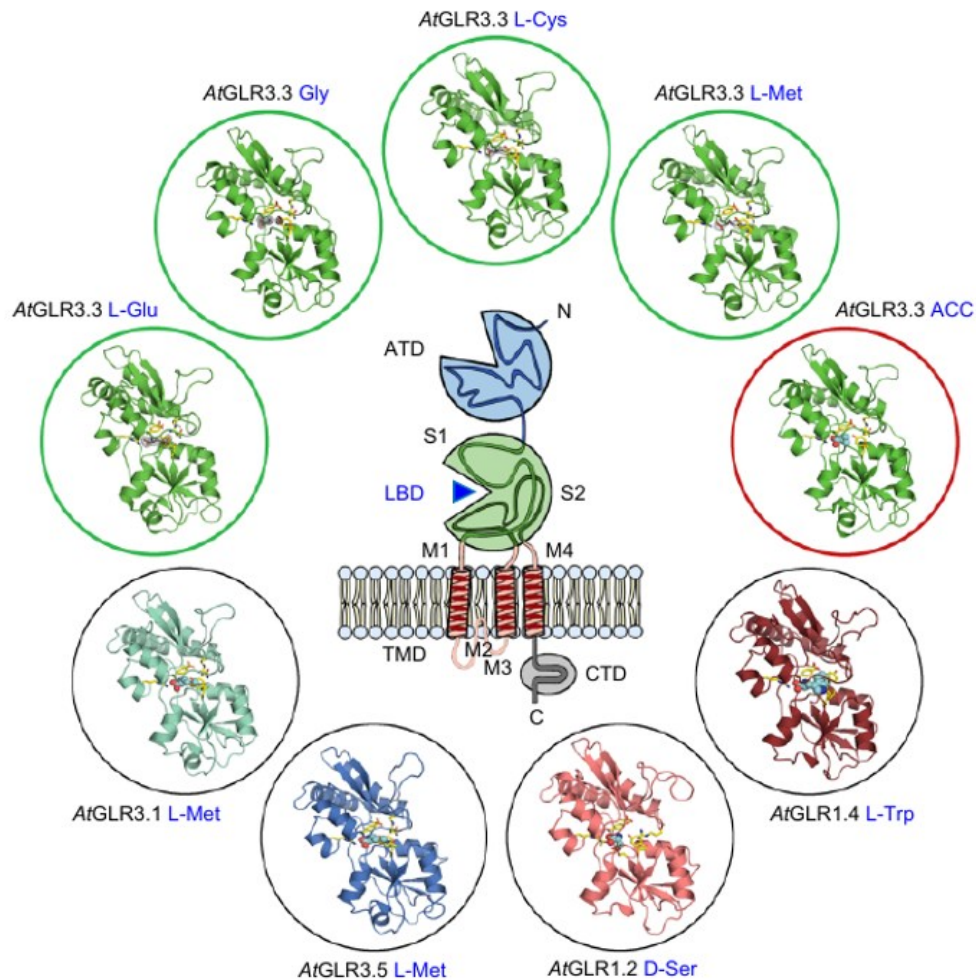


Figure 4 - Predicted architecture of plant glutamate receptor-like channels (GLRs). The schematic drawing shows the predicted membrane structure of a single GLR subunit. ATD amino-terminal domain, LBD ligand-binding domain, M1-4 membrane helices, CTD cytoplasmic tail. The four upper green-lined circles show the structures in ribbon representation of the AtGLR3.3-LBD bound to different ligands (from the left to the right: L-Glu, Gly, L-Cys and L-Met). The red-lined circle shows that the same AtGLR3.3-LBD structure is in principle able to accommodate the Aminocyclopropane-1-carboxylic acid (ACC) ligand without any obvious steric hindrance. The remaining four bottom black circles show homology modelling, based on the above-mentioned AtGLR3.3-LBD structure. From Alfieri et al., 2020

Recent studies have provided the first crystal structures of GLR LBDs (Alfieri et al., 2020; Gangwar et al., 2020, 2021; Green et al., 2021) showing a clear resemblance of iGluRs organization with a bilobed binding domain capable of accommodating multiple ligands. Our group investigated the AtGLR3.3 LBD in complex with four AAs, L-Glutamate, Glycine, L-Cysteine, and L-Methionine, and identified key residues, evolutionarily conserved, involved in ligand binding and stabilization: arginine 550 (R550) and aspartic acid 543 (D543). Our prediction suggested that R550 could directly interact with the ligand carboxyl group, while D543 stabilizes the LBD and traps the ligand inside the bilobed structure. Complementing the *glr3.3-1* knock out mutant with GLR3.3 variants mutated in their LBD for either of these key residues provided support for our hypothesis: GLR3.3_{D543G}

exhibited a significantly reduced activity compared to the GLR3.3_{WT} in response to exogenous L-Glutamate treatment, while GLR3.3_{R550N} showed no response (Grenzi et al., 2023).

In 2021, Green and colleagues were able to obtain, by means of Cryo-EM the first complete structure of a plant GLR, *AtGLR3.4* (Green et al., 2021). As anticipated, the subunits arrange in a tetrameric structure, similarly to iGluRs, but the channels show some key distinctions from its animal homologues. Moreover, a specific feature of GLR3.4 is the ability to bind GSH at the ATD level, and that this binding modulates its activity when expressed in COS-7 mammalian heterologous system (Burada et al., 2020; Green et al., 2021).

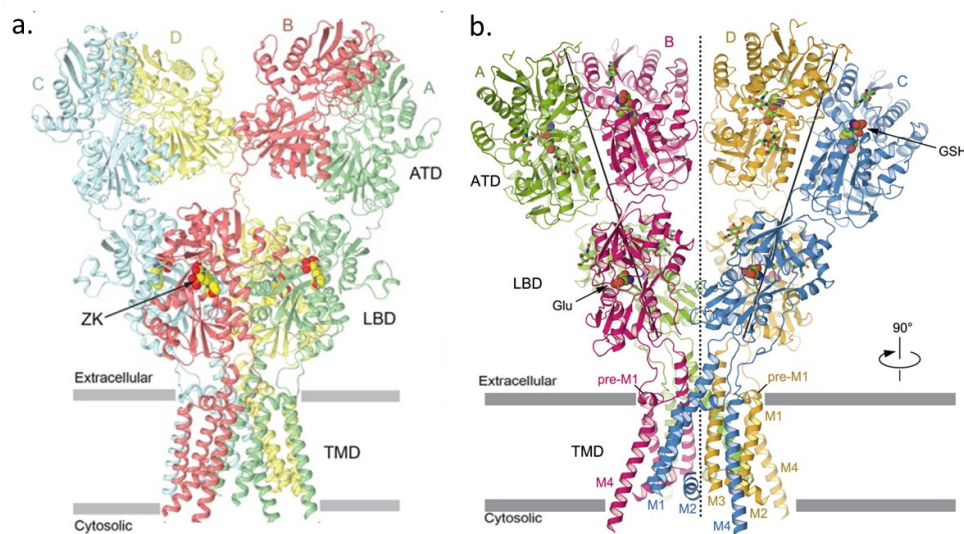


Figure 5 - Overall architecture of homo-tetrameric GluA2 AMPAR and GLR3.4. (a) Crystal structure of a homo-tetrameric AMPAR composed of GluA2 subunits in the closed state, bound to the competitive antagonist ZK200775. (b) homo-tetrameric structure of GLR3.4 in the closed state, bound to molecules of GSH and L-Glu. Each of the four subunits is coloured differently: green (A), red (B), blue (C), and yellow (D). ATD, Amino-Terminal Domain; LBD, Ligand Binding Domain; TMD, Transmembrane Domain. From Twomey & Sobolevsky (a), 2018 and Green et al., 2021 (b)

Another mechanism by which iGluRs are regulated is the composition of the channel pore: iGluRs, like GLRs, organize in tetramers to construct a functional pore, but they often hetero-tetramerize to modulate pore selectivity and activity (Traynelis et al., 2010). This property has been predicted also for some plant GLRs isoforms: For instance, Arabidopsis GLR3.4 and GLR3.2 have been found to colocalize in close proximity in mammalian HEK293 cells and *Nicotiana benthamiana* (Vincill et al., 2013), while GLR1.1, GLR2.9, GLR3.2 and GLR3.4 have been shown to interact with multiple subunits in yeast cells (Price et al., 2013). Kong et al., (2016) suggested that GLR3.1 and GLR3.5 form a heteromeric channel specifically activated by L-Met in stomata guard cells. Nevertheless, a deeper analysis aimed at better defining whether the formation of heteromeric plant GLR channels occurs lags behind.

2.9. Putative interactors of GLR3.3

A significant portion of my PhD project focused on study the putative role of the Arabidopsis GLR3.7 isoform to interact and regulate the activity of the GLR3.3. Certainly, genetics analyses previously suggested a role of GLR3.6 in regulating the activity of GLR3.3. Mousavi and colleagues reported that whereas the *glr3.3* mutant showed an impaired responses to wounding, including reduced electrical signal propagation and JA accumulation, this was further impaired in the double mutant *glr3.3/glr3.6* (Mousavi et al., 2013). An independent study also showed a reduced propagation of Reactive Oxygen Species (ROS) following wounding in *glr3.3/glr3.6* double mutant (Lew et al., 2020). This could suggest a possible direct relationship between the two proteins, but further investigation revealed that GLR3.3 and GLR3.6 display distinct expression patterns in adult plants vascular bundles: GLR3.3 localizes in phloematic companion cells, while GLR3.6 is restricted to xylem contact cells (Nguyen et al., 2018). This observation raises doubts about the formation of a heteromeric GLR channel in these tissues.

The selection of GLR3.7 for study was guided by an initial *in silico* analysis aimed at identifying the Clade III GLRs most ubiquitously expressed in the root tip meristematic tissues of Arabidopsis. This analysis revealed that *GLR3.3* and *GLR3.7* were among the most ubiquitously expressed GLRs (Doccu PhD thesis). Therefore, we hypothesize that GLR3.7 could potentially be an interactor of GLR3.3.

Among the few studies on GLR3.7, it was demonstrated to be involved in salt stress perception. The mutant allele *glr3.7-2* shows a reduced seed germination rate and a reduced $[Ca^{2+}]_{cyt}$ increase in seedlings following salt stress compared to WT (Wang et al., 2019). Recently, it was also reported that *GLR3.7* expression is regulated by Abscissic Acid (ABA) (Chen et al., 2021), a phytohormone important for seed development and stomatal closure (Cheng et al., 2014; Taylor et al., 1981). Notably, seed germination of *GLR3.7* overexpressing lines was enhanced under ABA treatment compared to WT (Chen et al., 2021). These pieces of information suggest a potential role of GLR3.7 in perception and response to the osmotic status of the plant, an ability that could be important during long-distance stress signaling mediated by pressure waves.

GLR3.7 also harbours, in its C-terminal domain, a calcium-dependent phosphorylation site, Ser860, fundamental for its interaction to 14-3-3 ω proteins, a class of proteins widely recognized for their role in the regulation of protein activity, localization, and association in complexes (Sehnke et al.,

2002). Notably, 14-3-3 ω proteins have also been characterized as regulators of NMDA in animals (Chen & Roche, 2009), further supporting the common ancestry of iGluRs and GLRs.

To determine if GLR3.7 has a direct relationship with GLR3.3, the most effective approach would be to investigate its impact on GLR3.3-dependent responses. However, how do we reliably study such responses?

2.10. Studying Ca^{2+} dynamics through *in vivo* imaging

As already mentioned, GLRs play a crucial role in long-distance signaling due to their activity as cation-permeable channels. Their cation selectivity often favours Ca^{2+} as the primary ion (Tapken & Hollmann, 2008; Vincill et al., 2012; Wudick, Portes, et al., 2018). Therefore, studying Ca^{2+} homeostasis provides insights into GLR activity, but what are the main techniques used to date?

Ca^{2+} imaging research gained momentum with the development of Ca^{2+} -sensitive dyes by Roger Tsien (Tsien, 1980). These dyes offer several advantages, including rapid application and compatibility with a wide range of organisms, but have their limitations. First they require loading, subject to technical variability, second, it's difficult to finely control the subcellular localization of the chemical, as the cell itself can compartmentalize or extrude these molecules, and last, the loading itself can cause responses in the sample, in some cases due to the die strong buffering capacity (Grenzi, Resentini, et al., 2021). A more reliable approach for Ca^{2+} imaging emerged with Genetically Encoded Calcium Indicators (GECIs) (Pérez Koldenkova & Nagai, 2013). Early in the development of GECIs, researchers sought to obtain a stable and reliable indicator for reporting Ca^{2+} concentrations *in vivo*, the Yellow Cameleon (Miyawaki et al., 1997). This genetically encoded Ca^{2+} indicator is based on the Forster Resonance Energy Transfer (FRET). FRET is a phenomenon between two fluorescent proteins where energy is transferred from one fluorescent protein, called the donor, to another fluorescent protein, called the acceptor. This event is quantifiable and dependent on two parameters, the spectral properties of the two proteins fluorophores and the distance: The emission spectrum of the donor must overlap with the excitation spectrum of the acceptor, and the transfer is more efficient when the two fluorophores are closer together.

In the Yellow Cameleon, the donor is a Cyan Fluorescent Protein (CFP), while the acceptor is a Yellow Fluorescent Protein (YFP). The two subunits are connected by a Calmodulin (CaM), able to bind Ca^{2+} , and a Calmodulin Binding peptide M13. The binding of Ca^{2+} by CaM cause an interaction between the CaM and M13 subunits causing the two fluorescent proteins to move closer together. This reduces the distance between the two fluorophores, increasing the efficiency of FRET: If the

CFP is excited, it will emit less photons and pass its energy to the YFP that will increase its photon emission. The readout of this sensor is based on the ratio between the emission of YFP and CFP, so the Yellow Cameleon has been defined as a Ratiometric FRET calcium indicator. This indicator, and its later iterations like the Yellow Cameleon 3.6 (YC3.6), possess some key advantages. First, ratiometric measurements is independent from protein abundance, meaning that the Ca^{2+} levels can be accurately measured even if the expression level of the indicator is not very high. Ratiometric measurements are also rather independent of acquisition focus and slight changes in pH, making them less sensitive to experimental conditions than other types of Ca^{2+} indicators (Grenzi, Resentini, et al., 2021). While they are useful tools, ratiometric indicators like YC3.6 have their limitations, like a wide spectral coverage, making it difficult to pair multiple indicators in a single experiment, lower dynamic range upon ligand binding, and the need for a specialized acquisition setup able to quantify FRET.

A different class of Ca^{2+} indicators was later developed to overcome these limitations, Intensiometric indicators. These proteins are made of a single fluorescent protein linked to a sensitive domain like the one used in the Cameleon. As an example, GCaMP3 and R-GECO1 are similar intensiometric sensors that feature a central fluorescent protein (GFP and circularly permuted mApple, respectively) and two terminal domains, a C-terminus CaM, and an N-terminus M13 peptide. Upon Ca^{2+} binding, these domains undergo a strong conformational change, resulting in a drastic change in the fluorescence emission spectra (Tian et al., 2009; Zhao et al., 2011).

The most interesting property of intensiometric indicators is that they are characterized by a very high dynamic range, meaning that upon Ca^{2+} binding they show a dramatic increase of the fluorescence emitted. Moreover, since they are based on a single fluorescent protein, its use can be coupled with other sensors with not overlapping excitation and emission spectra, thus allowing to perform multiparameter *in vivo* imaging. However, intensiometric indicators are more sensitive to experimental conditions (acquisition focus, pH, expression level) than ratiometric FRET indicators.

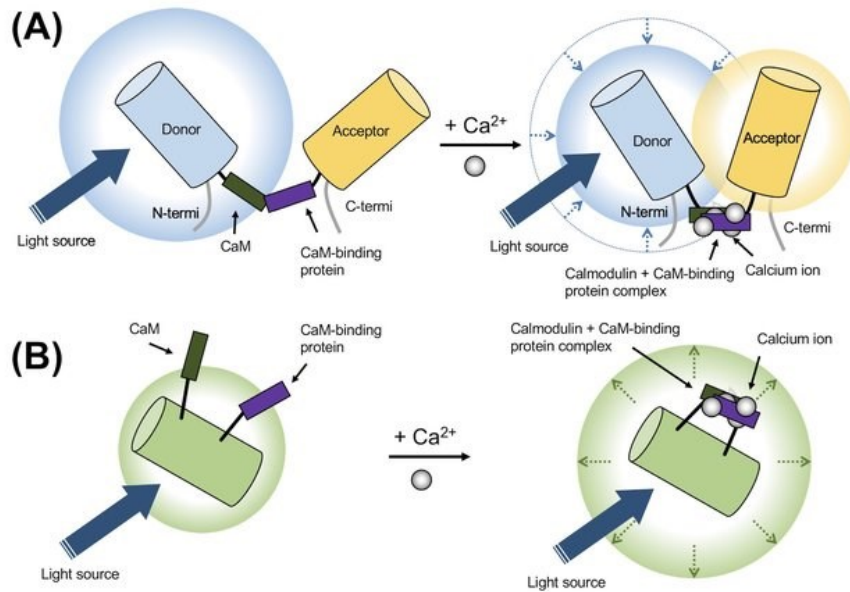


Figure 6 - Genetically encoded calcium indicators (GECI). (A) Fluorescence resonance energy transfer (FRET)-based GECI like the ratiometric Yellow Cameleon. Calcium ion binding enables approaching of donor with acceptor to induce FRET. (B) Single-fluorophore GECI like the intensimetric GCaMP. Calcium ions bind to an indicator that causes conformational changes leading to an increase of emitted light at fluorescence wavelengths. CaM, calmodulin; termi, terminal. From Oh et al., 2019.

This array of indicators made possible *in vivo* analysis of multiple parameters in close-to-physiological conditions in plants.

2.11. Arabidopsis GLR3.7 as a putative regulator of GLR3.3

As previously reported, GLR3.3 is required to mediate the AA-induced cytosolic Ca^{2+} increase (Alfieri et al., 2020; Qi et al., 2006; Stephens et al., 2008; Alfieri et al., 2020) playing a pivotal role in the propagation of long-distance electrical and Ca^{2+} signals induced by mechanical wounding, insect chewing, and osmotic stress (Bellandi et al., 2023; Grenzi et al., 2023; Mousavi et al., 2013; Nguyen et al., 2018; Toyota et al., 2018). Notably, our group reported that GLR3.3, through its LBD, binds several AA with varying, and that this binding is necessary to observe a cytosolic Ca^{2+} increase in root tip cells and long-distance Ca^{2+} waves in response to leaf mechanical damages (Alfieri et al., 2020; Grenzi et al., 2023). While the significant role played by GLR3.3 is clearly acknowledged, we explored the involvement of the GLR3.7 isoform. To test this hypothesis, we assessed if GLR3.7 may play a role in the GLR3.3 dependent responses.

2.11.1. GLR3.7 acts as negative regulator of GLR3.3-mediated AAs induced Ca^{2+} response in *Arabidopsis* root tip cells

We tested the AA-induced cytosolic Ca^{2+} increase in root tip cells of two *GLR3.7* independent mutant alleles, *glr3.7-1* and *glr3.7-2* expressing the Ca^{2+} sensor NES-YC3.6 (Krebs et al., 2012). In particular, we evaluated the response to 1 mM of 7 AAs (L-Cys, L-Glu, L-Ala, Gly, L-Ser, L-Asn, L-Met) known to induce GLR3.3 dependent $[\text{Ca}^{2+}]_{\text{cyt}}$ increase (Alfieri et al., 2020) (Fig 7.b).

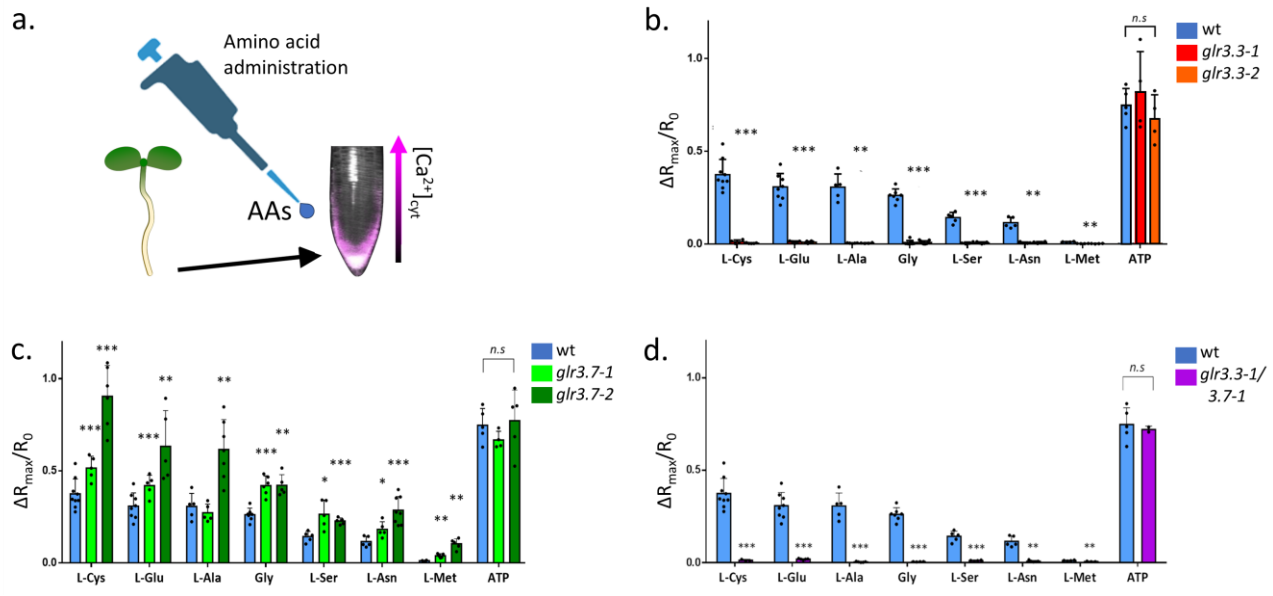


Figure 7 – *Arabidopsis* GLR3.3 dependent root tip sensitivity to exogenous amino acid administration. (a) schematical representation of the experimental setup. Maximal relative amplitude of cpVenus/CFP ratio as $\Delta R/R_0$ in the *glr3.3-1* and *glr3.3-2* (b), *glr3.7-1* and *glr3.7-2* (c) and *glr3.3-1 / glr3.7-1* (d) background. ATP administration was used as a positive control for $[\text{Ca}^{2+}]_{\text{cyt}}$ increase after exogenous administration of an elicitor: $n \geq 4$; error bars $\pm$ Standard Deviation; ns: not statistically significant, ** $p < 0.005$, *** $p < 0.0005$ (Student *t* test).

We then quantified the maximum $[\text{Ca}^{2+}]_{\text{cyt}}$ increase of WT and *glr3.7* mutant alleles, observing that both alleles, differentially from the *glr3.3* mutants, did not only show to properly respond to all tested AA, but the responses were exacerbated with maximum $[\text{Ca}^{2+}]_{\text{cyt}}$ increases that were higher compared to the WT (Fig 7.c). To determine whether the role of GLR3.7 is epistatic to GLR3.3 we generated a *glr3.3-1 / glr3.7-1* double mutant expressing the NES-YC3.6 and subjected the seedling to exogenous AAs. Similarly, to the single *glr3.3* mutant alleles, the double mutant did not show any $[\text{Ca}^{2+}]_{\text{cyt}}$ increase upon AA administration (Fig 7.d). This result suggests that, genetically, the GLR3.7 is a negative regulator of GLR3.3 activity in root tip cells.

2.11.2. GLR3.7 is a negative regulator of GLR3.3 mediated long-distance burning-induced responses

In Grenzi et al., 2023, we showed that local burning of one rosette leaf triggers a systematic response, propagating all the way to the floral tissues, where particularly in the flower receptacle, a clear increase in $[Ca^{2+}]_{cyt}$ is observed. Importantly, the rise of $[Ca^{2+}]_{cyt}$ is again, strictly dependent of GLR3.3 activity. To define if GLR3.7 may also have a role in the propagation of long-distance signals we performed the “burning experiment”, with all *glr3.7* mutant alleles. To do so, we applied a local burning with a lighter to one of the rosette’s leaves of *glr3.7-1* and *glr3.7-2* mutant backgrounds, expressing the NES-YC3.6, and analysed the $[Ca^{2+}]_{cyt}$ dynamics in the flower receptacle cells (Fig. 8.a) as reported in Grenzi et al. (2023).

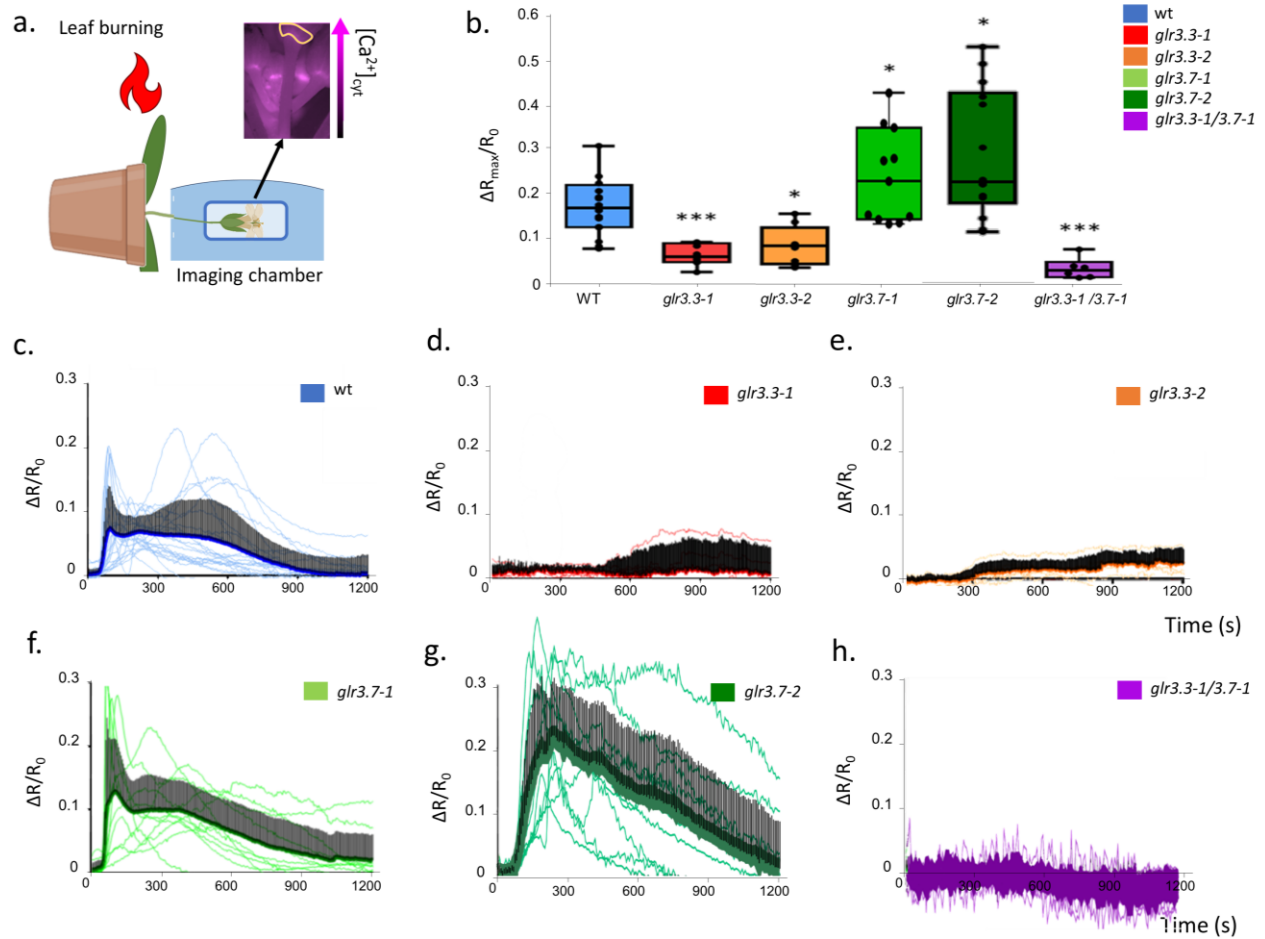


Figure 8 - Arabidopsis GLR3.3 dependent long-distance signaling of local burning stress. (a) schematical representation of the experimental setup. Maximal relative amplitude of cpVenus/CFP ratio as $\Delta R/R_0$ in the *glr3.3-1* and *glr3.3-2*, *glr3.7-1* and *glr3.7-2* and *glr3.3-1/3.7-1* background (b). $n \geq 4$; error bars $\pm$ Standard Deviation; ns: not statistically significant, ** $p < 0.005$, *** $p < 0.0005$ (Student *t* test). Representative curves of the cpVenus/CFP normalized ratio changes ($\Delta R/R_0$) acquired in response to the burn of one rosette leaf applied at 60 s from the beginning of the measurement (c-h).

The leaf burning induced a clear $[Ca^{2+}]_{cyt}$ increase in both WT and *glr3.7* mutant alleles (Fig. 8.c and 8.f-g), with these latter showing higher maximum $[Ca^{2+}]_{cyt}$ peaks compared to the WT (Fig 8.b). Similarly, to the root tip cells, even in the long-distance response where the *glr3.3* mutants do not show any $[Ca^{2+}]_{cyt}$ increase (Fig 8.d-e) the *glr3.7* mutants show an exacerbated one. To again determine whether the role of GLR3.7 is epistatic to GLR3.3 we also assessed the response of the double *glr3.3-1 / glr3.7-1* mutant to the leaf burning. Again, the double mutant response was almost abolished (Fig 8.h), imitating the single GLR3.3 mutants.

Comprehensively, these results indicate that GLR3.7 is a negative regulator of both the AA and burning induced $[Ca^{2+}]_{cyt}$ increase which primarily depend on the GLR3.3 activity.

Based on these genetic pieces of evidence, we wondered how GLR3.7 can exert its regulatory role on GLR3.3 activity, which could be dependent on a direct interaction between the two proteins (i.e. formation of an heteromeric channel) or due to other mechanisms.

3. Short overview of other works carried out during the PhD

Throughout my doctoral studies, I engaged in extensive research pertaining to the study of GLRs. In addition to this, I actively collaborated with other laboratories on diverse projects. These projects ranged from innovative methodologies to the creation of essential instruments tailored for conducting imaging experiments on adult plants, paralleling those applied in growth studies. Here, I briefly summarize the different works.

3.1. Non-invasive *in planta* measurements of H₂O₂ and glutathione redox potential with fluorescent roGFP2-based sensors

Inspired by the clear stress-driven propagation of Ca²⁺ waves in Arabidopsis plants, we sought to expand our analysis to include other crucial second messengers, such as ROS. Characterizing the interplay between Ca²⁺ and ROS needs at first selective, sensitive, and reliable sensors. While Ca²⁺ features a variety of dependable genetically encoded sensors (Grenzi et al., 2021), ROS remain elusive: these molecules exhibit a fast half-life and the selection for highly selective sensors has proven challenging. Currently, a widely used approach uses fluorescent chemical dyes (e.g. H₂DCF-DA), but these methods suffer from the limitations of dyes, already discussed in the introduction. Indeed, the need for a better ROS sensor led to the development of redox sensitive genetically encoded indicators.

In vivo imaging employing genetically encoded ROS and redox sensors *in planta* has evolved and diffused in the scientific community (Meyer et al., 2007; Schwarzländer et al., 2008; Schwarzländer et al., 2016). In particular, redox Green Fluorescent Protein (roGFP)-based sensors have been designed with the intention of measuring both the redox potential of glutathione pool as well as H₂O₂ synthesis (Gutscher et al., 2008; Meyer et al., 2007; Nietzel et al., 2019). Their functioning is akin to what already treated in the main introduction to Ca²⁺ imaging. Basically, these sensors are based on a modified GFP, where two cysteine residues have been inserted in adjacent β -strands on the surface of the protein β -barrel (Dooley et al., 2004; Meyer & Dick, 2010; Schwarzländer et al., 2016). In the cellular environment roGFPs are in a redox potential equilibrium with the glutathione couple (GSSG/2GSH), in a reaction catalysed by glutaredoxin (Grx). An increase of oxidative stress can be transferred, through Grx, to these two cysteines form a disulfide bond that induces a structural conformational change that influences the chromophore fluorescence. This fluorophore features a peculiar property, it possesses two main absorption peaks (at 405 and 488 nm, respectively) which in turn emit at the same emission peak (around 510 nm). The conformational change affects the quantum

yields (QY) of the two absorption peaks in an opposite way, leading to a ratiometric response (Meyer et al., 2007). Further iterations of this sensor aimed at better reliability and selectivity. The fusion of a human Grx1 to the roGFP2 made a 1:1 proportion between the roGFP2 sensor and its main intracellular catalyst of redox potential transfer, making the Grx1-roGFP2 indicator independent on subcellular concentration of Grx. Another evolution of the roGFP2 increased its specificity for H₂O₂, the most abundant and stable form of ROS (Apel & Hirt, 2004; Das & Roychoudhury, 2014). This sensor, roGFP2-Orp1, features the yeast Orp1 peroxidase (Orp1) which has an intrinsic capacity to act as a specific H₂O₂-dependent protein thiol oxidase (Gutscher et al., 2009). In this way, when sensing H₂O₂, the Orp1 subunit oxidize the roGFP2 (forming of the disulfide bridge), increasing its selectivity to H₂O₂ alone.

In this work we aimed to provide the community with an easy and straightforward protocol to reliably measure redox potential changes using the battery of roGFP2 based sensors.

3.2. Mitochondria-derived reactive oxygen species are likely the primary trigger of mitochondrial retrograde signaling in Arabidopsis

As already introduced, the cytosol environment is heavily influenced by subcellular compartments, and second messengers' mediated responses are not an exception. An example of how organelles can generate and modulate responses to external cues and influence the rest of the cell is mitochondrial retrograde regulation (MRR). This feedback regulation allows for a joint effort in maintaining cellular homeostasis from the nucleus and the mitochondria, also involving second messengers. Being the centre of the respiration pathways, mitochondria are prone to the production of Reactive Oxygen Species (ROS). ROS existence is deeply intertwined with an aerobic metabolism, as natural by-products of electrons transport in photosynthesis and respiration (Das et al., 2015). As highly reactive and oxidant chemicals, ROS are recognized as potentially dangerous molecules for the delicate cellular environment, but can also be necessary for the signalling purposes (Apel & Hirt, 2004; Rhee, 2006).

As the name states, ROS are highly reactive and characterized by a short half-life. This property should limit their extent of movement and ability to transmit signals, but interestingly, ROS maintain a crucial role in long distance signalling of abiotic stresses (wounding, cold, heat, light and salinity) (Miller et al., 2009), in coordination with Ca²⁺.

In Khan et al., 2024, we show that mitochondrial ROS are one of the main triggers of MRR in plants: Blocking the activity of electron transport chain (at the level of complex III) with Antimycin A is a

strong inducer of MRR. Its effect rapidly propagates to the rest of the cell. It induces a rapid decrease in $[\text{MgATP}^{2-}]_{\text{cyt}}$ inducing a proportional acidification of the cytosol, possibly due to the reduction of proton pumping across the membrane caused by low ATP availability, and increase of NADH/NAD⁺ ratio, indicative of the inability of mitochondria to consume NADH. Antimycin A also induced an increase of $[\text{Ca}^{2+}]_{\text{cyt}}$, $[\text{H}_2\text{O}_2]_{\text{cyt}}$ and an increase in the expression of different oxidative stress responsive marker genes, like H₂O₂ responsive gene 1 (HRG1). Similar effects were obtained with Oligomycin, an inhibitor of mitochondrial ATP synthase, but also with Monofluoroacetate, an inhibitor of the tricarboxylic acid cycle and causing citrate accumulation.

To test whether the MRR was induced by mitochondrial ROS leaking into the cytoplasm, the same analyses performed in response to the previously reported chemicals were repeated by treating the seedlings with Mito-Paraquat, a drug able to specifically induce mitochondrial ROS accumulation. Notably, Mito-Paraquat induced MRR without inducing any effect on cytosolic parameters like $[\text{MgATP}^{2-}]_{\text{cyt}}$, pH_{cyt} , NADH/NAD⁺ ratio, $[\text{Ca}^{2+}]_{\text{cyt}}$ and $[\text{H}_2\text{O}_2]_{\text{cyt}}$.

This information is particularly interesting: mitochondrial oxidative stress can be perceived by the rest of the cell, inducing MRR, without directly affecting the cytosolic second messengers' homeostasis.

3.3. AMEBaS: Automatic Midline Extraction and Background Subtraction of Ratiometric Fluorescence Time-Lapses of Polarized Single Cells

Imaging is a powerful tool, often so powerful that the main bottleneck in data extraction is none other than the analysis of the acquired image. The development of multiple imaging supports and cutting-edge imaging technologies allow us to extrapolate more information from our samples and while data extraction is one of the main activities of a microscopist, it often falls short of reliability due to the intrinsic bias in choosing the right parameter to sample. Additionally, when working with live specimens, data analysis can be complex due to sample movement and multiple regions of interest (ROI). During my PhD I had the pleasure to collaborate with researchers from different fields to design and tailor post processing platforms for a more reliable data extraction and analysis. From a collaboration with the group of Professor Andrea Bassi, from the Physics department of the “Politecnico di Milano”, we developed a customized data analysis pipeline, a python based Napari plugin, which provide a reliable and user-friendly approach to multiple ROI registration analysis (Roi Registration, <https://www.napari-hub.org/plugins/napari-roi-registration>). This useful tool proved

fundamental in deciphering $[Ca^{2+}]$ oscillations in “moving ROIs”, like growing root hairs and pollen tubes.

From this collaboration also stemmed a joint work with Dr. Roberto M Cesar Jr, from Institute of Mathematics and Statistics of the University of São Paulo, which developed a computational pipeline to automate and quantify the spatiotemporal behaviour of single cells using, as a model of cell polarity, our data from $[Ca^{2+}]_{\text{cyt}}$ evaluation during root hair growth. These tools are fundamental in reducing time spent during analysis and biases based on the operator.

3.4. 3D printing in “plant molecular imaging”

During my PhD I focused on understanding how plants perceived and responded to a multitude of stresses. This topic poses unique challenges due to the delicate sensitivity of plants to manipulation. It is crucial to develop better protocols and solutions to expose plants to conditions simulating environmental stresses, enabling us to investigate their responses and adaptations, and while various companies provide readily available solutions but that often don't reach the level of customization required to this end. In such an environment it is important to be able to quickly prototype and produce tailored solutions to study multiple parameters or focus onto a specific aspect of plant homeostasis. To address this issue, I applied my knowledge of 3D printing to design reliable tailor-made solutions to study plants response to environmental challenges.

3D printing is a widely used technology that in the last years has paved the way for a much more sustainable, more cost-effective, more customizable, and faster prototyping and design of products. It has been employed in multiple fields of life sciences from food (Handral et al., 2022), to medicine (Aimar et al., 2019). The flexibility and versatility of 3D printing thus represents a valuable approach to improving experimental designs, in particular those involving microscopy.

Among the main 3D printing technologies, the most diffused are the following: Fused Deposition Modelling (FDM) is the cheapest and most widespread technology: It operates by deposition of molten material in layers to create a three-dimensional object. Stereolithography printing (SLA) is slightly more expensive, but far more precise than FDM: It exerts UV light to polymerize a liquid resin in layers, one on top of the other. Selective Laser Sintering (SLS) fares in the span of high-end industrial grade 3D printing by exploiting high-power lasers to fuse particles of material into layers with a higher range of materials compared to SLS and FDM technologies. Selective Laser Melting (SLM) is a similar technology to SLS, but focus on metal 3D printing, requiring even higher laser power and industrial machinery for post-processing. In our case, FDM and SLA are the most

appealing: they are both cheap and readily available technologies that do not require extensive treatment and can widely improve the design of experimental approaches. With knowledge of 3D design, we were able to develop new supports for specimen growth, innovative solutions for *in vivo* imaging and treatment.

This technology was fundamental for performing some imaging analyses aimed at working with adult plants in their standard growth conditions.

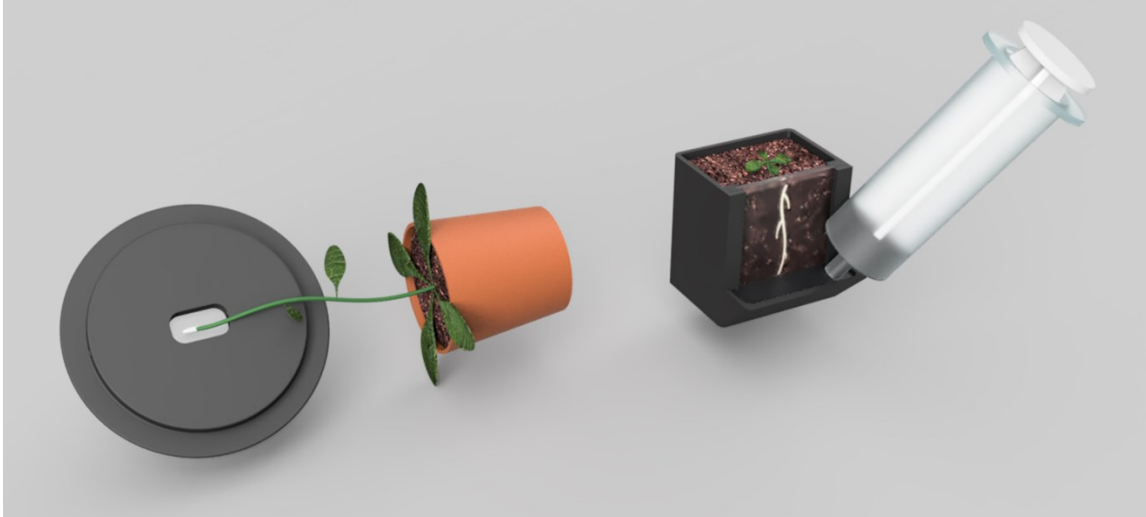


Figure 9 - High resolution rendering of two imaging supports featured in this thesis. On the left an imaging chamber for *in vivo* imaging of floral organs, on the right a growing chamber for *in vivo* imaging of leaf and root tissues of adult plants.

4. Aim of the thesis

Plants are complex organisms that possess remarkable resilience and adaptability to diverse environmental challenges. Their ability to perceive external stimuli is, therefore, crucial to trigger a tailored response. More so, plants need to communicate the local perception of a stimulus (e.g. roots) to other systemic organs or tissues. One protein family, GLR, has been shown to be required to allow the propagation of electrical and Ca^{2+} long distance signals triggered by the perception of different stresses (Wudick et al., 2018; Grenzi et al., 2022). Thus, their nature as cation permeable channels makes them essential for the generation of surface wave potentials (SWPs) and increases in cytosolic calcium concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) (Mousavi et al., 2013; Nguyen et al., 2018).

Among these proteins, GLR3.3, stands out as a key GLR isoform involved in long-distance signaling in Arabidopsis (Mousavi et al., 2013; Nguyen et al., 2018; Grenzi et al., 2023). It is activated by the binding of AAs, such as L-Glu (Alfieri et al., 2020), that accumulates in the apoplast in response to wounding, burning, herbivory and osmotic stress stimuli (Bellandi et al., 2023; Grenzi et al., 2023). This activation triggers the influx of Ca^{2+} into the cytosol, activating downstream signaling cascades that allow stress response and adaptation (Bellandi et al., 2023; Grenzi et al., 2023; Mousavi et al., 2013).

To better understand the regulatory mechanisms of such a key player in long-distance plant stress responses, I devoted most of my PhD to characterize the potential regulatory oligomerization of GLR3.3.

Among the vast GLR family in Arabidopsis, an interesting candidate piqued our interest, GLR3.7. GLR3.7 is a GLR isoform that has been implicated in salt stress signaling and Absciscic Acid perception (ABA) as well as in pollen tube development (Michard et al., 2011; Chen et al., 2021; Wang et al., 2019). Our preliminary analyses (Doccia, Grenzi et al., unpublished), including root tip response to exogenous AAs administration and distal response to a local burning stress, revealed that GLR3.7 plays a negative regulatory role on GLR3.3-mediated responses. This suggested us that GLR3.7 may contribute to the plants responses, possibly through a direct interaction with GLR3.3.

To further elucidate the interaction between GLR3.3 and GLR3.7 and unravel how they regulate each other's activity, I defined the following aims:

- 1) To investigate the expression pattern of GLR3.3 and GLR3.7 through a translational GUS reporter assay in *Arabidopsis thaliana*;
- 2) To study GLR3.3 and GLR3.7 *in vivo* interaction by means of Fluorescence Lifetime Imaging (FLIM);

- 3) To investigate the possible interaction of GLR3.3 and GLR3.7, by means of a Co-Immunoprecipitation assay in the heterologous model system *Saccharomyces cerevisiae*;
- 4) To test *in vivo* the GLR3.7 effects of GLR3.3 in a simplified non-plant system;
- 5) To define the physiological role of GLR3.3 and GLR3.7 interplay during plant rehydration responses.

5. Materials and methods

5.1. Plant material and growth conditions

5.1.1. Plant strains

The main plant model used in this study is *Arabidopsis thaliana* ecotype Columbia 0 (Col-0), for transient transformation assays (e.g. Fluorescence Lifetime Imaging analysis) we also employed *Nicotiana benthamiana* tobacco plants.

5.1.2. Seed sowing and plant growth

Seed were sterilized via ethanol gradient sterilization: first a 15 min wash in 70% (v/v) ethanol, then washed two times in sterile conditions with 100% (v/v) ethanol and left to dry on sterile filter paper under the laminar flow hood. Once fully dried, seeds were plated on half strength Murashige and Skoog solid media (Murashige & Skoog, 1962) (Duchefa, <http://www.duchefa-biochemie.com/>) enriched of 0.1% (w/v) sucrose, 0.05% (w/v) MES monohydrate, pH to 5.8, and then 0.8% (w/v) plant agar (Duchefa, <http://www.duchefa-biochemie.com/>). After a 2 days stratification at 4°C, plates were transferred to Conviron GEN1000 SH plant growth chambers (Conviron, <https://www.conviron.com/>) under long day conditions: 16 h light/8 h dark cycles (long day), 100 $\mu\text{E}/\text{m}^2\text{s}$ cool white LED light, 22°C/18°C.

7 days after germination, seedlings were either used for analysis or transferred in soil and grown in Conviron GEN1000 SH plant growth chambers in long day conditions.

For soil grown plants, pots were filled with autoclaved potting compost and abundantly watered. Seedling were transferred and kept in high humidity by covering the pots with transparent plastic wrap. After 7 days plants were uncovered and grown to maturity.

Nicotiana benthamiana plants were grown in dedicated greenhouses under long day conditions: 16 h light/8 h dark cycles (long day), atmospheric light implemented with white incandescent lamps, 22°C/18°C.

5.2. Microbiology material and growth conditions

5.2.1. Bacterial strains

For plasmid cloning and purification we employed *Escherichia coli* DH5 α competent strain, while *Agrobacterium tumefaciens* strain GV3101/pMP90 was the strain of choice for plant transformation.

5.2.2. Yeast strains

Na⁺-hypersensitive mutant *Saccharomyces cerevisiae* G19 strain (Quintero et al., 1996) was used for the Co-Immunoprecipitation assay and yeast complementation assay.

5.3. Molecular biology methods

5.3.1. PCR conditions

A Go-Taq polymerase kit (Promega Corporation, <https://Promega.com>) was used for standard PCR protocols as following:

<10 ng DNA were mixed with 0.5 μ L of dNTPs 10 mM mix, 5 μ L of 5X Green Go-Taq Reaction Buffer, 0.1 μ L Go-Taq G2 DNA Polymerase 5 U/ μ L, 0.5 μ L forward/reverse primers 10 μ M and taken to a final volume of 25 μ L with ddH₂O.

For cloning, a High Fidelity Q5 kit was preferred:

<10 ng DNA were mixed with 1 μ L of dNTPs 10 mM mix, 10 μ L of 5X Q5 High-Fidelity DNA Polymerase Reaction Buffer, 1 μ L Q5 High-Fidelity DNA Polymerase 2 U/ μ L, 1 μ L forward/reverse primers 10 μ M and taken to a final volume of 50 μ L with ddH₂O.

5.3.2. mRNA extraction from plant tissues and cDNA library synthesis

To assess the expression level of the main GLR clade 3 components (*ATGLR3.1* AT2G17260, *ATGLR3.2* AT4G35290, *ATGLR3.3* AT1G42540.1, *ATGLR3.4* AT1G05200.1, *ATGLR3.5* AT2G32390, *ATGLR3.6* AT3G51480, *ATGLR3.7* AT2G32400) in WT, *glr3.7-1* and *glr3.7-2* background, we extracted total RNA from 7-day-old seedlings.

The LiCl-based total RNA extraction is fully described in (Verwoerd et al., 1989). Extracted material was then treated with DNase I (NEB, <https://international.neb.com>) to remove all traces of gDNA.

We then obtained a cDNA library from 0.5 µg of RNA through the LunaScript Reverse Transcriptase SuperMix Kit (NEB, <https://international.neb.com>).

5.3.3. Quantitative RT-qPCR analysis

The cDNA obtained was used as template for a quantitative Reverse Transcription PCR (RT-PCR) to indirectly assess the transcript abundance of the genes of interest. Quantification was performed via the iTaq Universal SYBR Green supermix (Bio-Rad Laboratories, <https://bio-rad.com/>) coupled with the CFX 96 Real-Time PCR detection system (Bio-Rad Laboratories, <https://bio-rad.com/>) in technical triplicates. Primers used are listed in Table 1.

cDNA levels were standardized relative to Tubulin (AT5G44340) and transcripts levels quantification were performed with the $2^{-\Delta\Delta Ct}$ method of the Bio-Rad CFX Maestro software v.4.0 (Bio-Rad Laboratories, <https://bio-rad.com/>). Values represent averages from three biological samples.

5.3.4. Molecular cloning and plasmid constructs – *pESC-LEU pGAL1::gGLR3.3-c-Myc / pGAL10::GLR3.7-FLAG*

As starting vector for a parallel and similar expression efficiency of GLR3.3 and GLR3.7 we chose the *pESC-LEU* yeast epitope tagging vector (Agilent technologies, <https://www.agilent.com/>). This construct is composed of a LEU2 gene for leucine autotrophy (to select transformed cells) and two multicloning sites, MCS1 and MCS2, under the pGAL1 and pGAL10 galactose inducible promoters, assuring parallel and similar expression levels of the two sites, and tagged with the c-Myc and FLAG tag respectively.

GLR3.7 (AT2G32400) was inserted in the MCS1 under the promoter pGAL1 and a FLAG tag (peptide sequence: DYKDDDDK) through enzymatic digestion. We amplified the GLR3.7 Coding Sequence (CDS) from WT col0 plants cDNA with a High Fidelity Q5 polymerase guided by AC597 and AC598 primers, which also inserted a NotI cutting site. The amplicons and vector were digested with the NotI enzyme and successively ligated with a T4 DNA ligase (NEB, <https://international.neb.com>).

GLR3.3 (AT1G42540.1) was inserted inside the MCS2 under the promoter pGAL10 and the c-Myc tag (peptide sequence: EQKLISEEDL) through a NEB builder-based procedure (NEB, <https://international.neb.com>). We first linearized the vector by enzymatic digestion in the MCS2 through the ApaI enzyme, then inserted through ligation the T4 DNA ligase GLR3.3 CDS, amplified

with a High Fidelity Q5 polymerase from WT col0 plants cDNA with AC595 and AC596 primers, which also inserted the sites needed for NEB builder recombination.

To check for a correct insertion of the genes into the host vector, making sure of the integrity of the CDS and the in-frame insertion of the gene ATG and tag peptide, we sequenced them.

5.3.5. Molecular cloning and plasmid constructs – pGLR3.7::gGLR3.7-GUS

To investigate the localization of GLR3.7 (AT2G32400) at different developmental stages and tissues, we employed a translational β -Glucuronidase (GUS) assay. We inserted the promoter and full genomic of GLR3.7 GUS gene into a pGreen0029 empty vector through enzymatic digestion.

First, we amplified the GUS gene with a High Fidelity Q5 polymerase and AC606-AC607 primers to insert BamHI and NotI restriction sites, then we opened the vector with the same enzymes and ligated the gene with a T4 DNA ligase.

The second step simultaneously inserted the promoter (980 bp before the ATG sequence) and the genomic sequence of GLR3.7, amplified with a High Fidelity Q5 polymerase and AC433-AC609 primers couple, inserting the needed XmaI restriction site. The pGreen0029-GUS vector was opened just before the GUS sequence with XmaI restriction enzyme, and the amplicon sequence was ligated in place with a T4 ligase.

To check for a correct insertion of the genes into the host vector, making sure of the integrity of the CDS and the in-frame insertion of the gene ATG and tag peptide, we sequenced them.

5.3.6. Molecular cloning and plasmid constructs – pB7FW 2.0 *p35s::GLR3.3-GFP* / pGPTVII *pUBQ10::GLR3.7-mCherry*

pB7FW2.0 *p35s::GLR3.3-GFP* was fully described in (Grenzi et al., 2023), while pGPTVII *pUBQ10::GLR3.7-mCherry* was kindly provided by Prof. Jörg Kudla, university of Münster, Germany.

5.3.7. Yeast transformation

Transformation solution 34% (w/v) PEG3500, 100 mM LiAc, 50 ng Carrier DNA, 600-800 ng Construct DNA.

To generate a construct for yeast expression and coimmunoprecipitation of GLR3.3 and GLR3.7 the two proteins were transformed in yeast strain G19 through a *pESC-LEU* vector. Yeast G19 cells were grown for two days on SC solid media composed of 0.69% (w/v) Yeast nitrogen base without aminoacids, 50 mM Succinic acid, 50 mM KCl, 0.069% (w/v) Yeast complete supplement, 1.5% (w/v) Agar and added with 2% (w/v) Glucose as the only carbon source. A full loop of cells was transferred to 2 mL tubes and diluted in 1 mL sterile water. It was then spun 1 minute at 4000 xg to remove the supernatant.

The cells obtained were resuspended in 1 mL LiAc 100 μ M and incubated 5 minutes at 30°C. After spinning the cells for 1 minute at 4000 xg we resuspended them in 350 μ L of transformation mix (34% (w/v) PEG3500, 100 mM LiAc, 50 ng Carrier DNA, 600-800 ng Construct DNA) and vortexed for 1 minute. The cells were then incubated for 20 minutes at 42°C. After this step, the cells were spun 1 minute at 4000 xg to remove the supernatant, washed in 1 mL ddH₂O, and pelleted for 1 minute at 4000 xg. The washed pellet was then resuspended in 200 μ L of sterile ddH₂O and plated on SC-LEU selective solid media composed of 0.69% (w/v) Yeast nitrogen base without aminoacids, 50 mM Succinic acid, 50 mM KCl, 0.069% (w/v) Yeast complete supplement dropout without Leucine, 1.5% (w/v) Agar and added with 2% (w/v) Glucose as the only carbon source.

5.3.8. DH5 α competent *E. coli* strain transformation

We used a freeze-thaw protocol to transform 50 μ L DH5 α competent *E. coli* cells with 100 ng of plasmid DNA, successively incubated in ice for 30 minutes. Heat shock was then induced to open cell membranes by transferring the mix to a 42 °C water bath for 45 seconds and then back in ice for 2 minutes. To allow cell recovery and resistance genes expression, the cells were then supplied with 500 μ L of fresh Luria-Bertani (LB) (1% (w/v) Peptone, 5% (w/v) Yeast extract, 5% (w/v) NaCl) liquid media and incubated at 37 °C for 1 hour. Transformed cells were then plated on selective solid LB media with 2% (w/v) Agar and incubated overnight at 37 °C.

5.3.9. Plasmid DNA extraction

Resistant colonies of DH5 α *E. coli* cells were inoculated in liquid LB broth with the appropriate selective antibiotics overnight at 37 °C under continuous shaking (0.1 xg). DNA was extracted from cells in exponential growth with the Monarch Plasmid Miniprep Kit (NEB, <https://international.neb.com>) through an alkaline lysis method.

5.3.10. GV3101 competent *Agrobacterium tumefaciens* strain transformation

Similarly to *E. coli*, we used a freeze-thaw protocol to transform 100 µL GV3101/pMP90 competent *A. tumefaciens* cells with 1 µg of plasmid DNA, successively frozen in liquid nitrogen. Heat shock was then induced to open cell membranes by transferring the mix to a 37 °C water bath for 5 minutes. To allow cell recovery and resistance genes expression, the cells were then supplied with 1 mL of Yeast Extract Peptone (YEP) liquid media (1% (w/v) Tryptone, 1% (w/v) Yeast extract, 5% (w/v) NaCl, pH 7.0) liquid media and incubated at 28 °C for 2 hours. Transformed cells were then plated on selective solid LB media with 2% (w/v) Agar and incubated two days at 28°C.

5.3.11. Transient expression in *Nicotiana benthamiana* leaves

For the FLIM analysis, we transiently expressed the *pB7FW 2.0 p35s::GLR3.3-GFP / pGPTVII pUBQ10::GLR3.7-mCherry* constructs in *Nicotiana benthamiana* leaves. As infection vector we employed *Agrobacterium tumefaciens* GV3101/pMP90 strain, carrying the construct, in parallel with the p19 expression enhancer system according to Waadt & Kudla, 2008, infiltrated in leaves from 5 weeks old plants. After 3-5 days the plants are ready for confocal imaging.

5.3.12. Generation of *Arabidopsis* transgenic plants

Transgenic plants were obtained by means of a floral dip approach, using *Agrobacterium tumefaciens* GV3101/pMP90 strain as infection vector. *Agrobacterium* cultures carrying the pGLR3.7::gGLR3.7-GUS construct were incubated overnight in 100mL YEP with appropriate selective antibiotics and successively pelleted at 7000 xg at room temperature (RT) for 15 minutes. After discarding the supernatant, we resuspended the pellet in 200 mL of Dip Solution (5% (w/v) Sucrose, 0.05% (v/v) Silwet L-77). Adult WT plants were dipped three times for 15 seconds each and left one full day at dark to induce *Agrobacterium* infection. After this step, the plants were grown in a growing chamber to full maturity to then collect the transformed seeds.

These seeds were then germinated in selective MS1/2 solid media with 50 mg/L Kanamycin to select only transformed plants.

5.4. Co-Immunoprecipitation assay

5.4.1. Cell growth and induction

Transformed G19 yeast strains were grown at 30 °C on solid SC-LEU, selective for our *pESC-LEU* plasmids described above, solid media with 2% (w/v) Glucose as the only carbon source.

Cells were collected from the solid media and inoculated in 20 mL of SC-LEU liquid media (with identical composition to the solid media, without agar) with 2% (w/v) Glucose as the only carbon source, grown at 30 °C to reach a suitable cell concentration for the induction step, around 1.00 Optical Density 600 nm (OD₆₀₀).

Before activating the Galactose inducible promoters, we had to remove any remaining Glucose, to force the yeast to use Galactose as main carbon source. To do so we washed the samples three times by pelleting the cells at 1500 xg for 3 minutes, removing the supernatant and washing the pellet with 15 mL of sterile double distilled H₂O (ddH₂O).

Then cells were resuspended in 50 mL SC-LEU liquid media with 2% (w/v) Galactose as the only carbon source and grown at 30 °C for around one day or until reaching around 3.5 OD₆₀₀. At this point cells were pelleted at 3500 xg for 10 minutes, the supernatant was removed and the pellet was stored at -80 °C.

5.4.2. Cell lysis

Each lysis step requires around 0.4g of cell pellet. The pellet was relocated into a 15 mL tube and diluted into 400 µL of Lysis buffer (50 mM TrisHCl pH 8, 150 mM NaCl, 5 mM EDTA pH 8, 0.4% (v/v) Triton X100, 50 mM NaF, 1 mM PMSF, 1 mM Na₃VO₄) with protease inhibitors to enhance protein stability (5 µg/ml Leupeptin and 10 µg/ml Chymostatin).

450 µL of lysis mix was incubated in ice 10 minutes while around 0.3 mg of 425-600 µm glass beads (Sigma-Aldrich, <https://www.sigmaaldrich.com/IT/it>) were soaked in lysis buffer. The beads were transferred to the lysis mix and cells were mechanically disrupted 10 times by alternating 1 minute of vortex and 1 minute of rest in ice.

The lysate was then separated from the glass beads and transferred to a 2 mL tube.

5.4.3. Co-Immunoprecipitation

The lysate sample was used fresh from the cell lysis step. We stored 100 μ L of the lysate as a "Loaded" control containing all proteins in our starting sample.

We added 50 μ L of anti-c-Myc μ MACS™ Epitope Tag Protein Isolation beads (Milteny Biotech, <https://www.miltenyibiotec.com/US-en/>) to 350 μ L of the lysate and incubated the mix at 4 °C for 30 minutes. Meanwhile, we soaked the Co-IP column with 200 μ L of washing buffer (50 mM TrisHCl pH 8, 5 mM EDTA pH 8, 50 mM NaF, 1 mM PMSF, 1 mM Na₃VO₄).

The column was then loaded into the μ MACS™ magnetic separator and filled with the tagged cell lysate. The solution flowing through the column at this stage was stored as "Unbound".

Remaining unbound proteins were washed from the column with two subsequent loading of 500 μ L washing buffer. Each flow-through was separately stored as "First Wash", "Second Wash", and "Third Wash".

To start the elution step, we loaded 20 μ L of preheated (at 60 °C) Laemmli elution buffer 1x (60 mM TrisHCl pH 6.8, 2% (v/v) SDS, 10% (v/v) Glycerol, 100 mM Beta-Mercaptoethanol, 0.025% (w/v) Bromophenol Blue) and incubated the column for 5 minutes. Column-bound proteins were detached in the last elution step by loading 80 μ L of Laemmli elution buffer preheated at 60°C 1x and the Flow-through was collected as "Eluted".

All stored samples, except the "Eluted", were solubilized by adding Laemmli elution buffer 2x (360 mM TrisHCl pH 6.8, 12% (v/v) SDS, 60% (v/v) Glycerol, 600 mM Beta-Mercaptoethanol, 0.15% (w/v) Bromophenol Blue) to reach a final concentration of elution buffer of 1x. All fractions were then incubated at 60°C for 30 minutes to conclude proteins solubilization and denaturation.

5.4.4. Microsomal fraction extraction

To assess the expression and accumulation of the GLR3.3 and GLR3.7 proteins in our yeast strains, we decided to parallelly extract their microsomal fraction to for better insoluble proteins detection. 1 g of yeast cells pellet was resuspended in 1 mL of lysis buffer (50 mM TrisHCl pH 8, 150 mM NaCl, 5 mM EDTA pH 8, 0.4% (v/v) Triton X100, 50 mM NaF, 1 mM PMSF, 1 mM Na₃VO₄). 800 μ L of the solution were centrifuged at 20000 xg at 4 °C for 1 hour and after removing the supernatant (soluble fraction), the resulting pellet was resuspended in 100 μ L of lysis buffer and 14 μ L of protease inhibitors solution (1 mg/mL Eupeptyn, 20 mM P-Aminobenzamide, 5 mg/mL Tosyl-Lysin, 25 μ g/mL soy Trypsin inhibitor) and incubated at 4 °C for 5 minutes. The resulting solution was mixed with

100 μ L of Solubilization buffer (4% (v/v) SDS, 3% (v/v) β MerCaptoethanol, 20% (v/v) Glycerol, 1 mM EDTA, 20 mM H_3PO_4 , pH 2.4) and incubated at 25 °C for 1 hour. Proteins were quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, <https://bio-rad.com/>) using a concentration scale of γ -Globulin as reference.

5.4.5. SDS-page and Western blot

We loaded a 10-wells 8% ExpressPlus Page gels (GenScript, <https://www.genscript.com/>) with 30 μ l of “Loaded”, “Unbound”, “First wash”, and “Eluted” fractions and a prestained protein standard broad range ladder (NEB, <https://international.neb.com>).

The proteins were separated via electrophoresis in a Tris-MOPS-SDS buffer (50 mM Tris Base, 50 mM MOPS, 3.5 mM SDS, 1 mM EDTA) running buffer first for 15 minutes at fixed voltage of 100 V to stack the proteins, then at 130 V, 0.1 A, for 60 minutes to separate them.

After the run the gel was used for a 0.45 μ m nitrocellulose transfer Western blot at 150 V, 0.150A for 2 hours in TB buffer 1x (50 mM Tris Base, 380 mM Glycin, 0.1% v/v SDS, 20% v/v Methanol). The western blot obtained was then blocked with 3% (w/v) BSA and 0.2% (v/v) Tween20 in TBS 1x (0.1 mM Tris-HCl pH 7.4, 0.75 M NaCl) overnight.

5.4.6. Immuno-decoration

To visualize the separated proteins, we employed tag-mediated immuno-decoration.

First, we washed the nitrocellulose membrane with TBS 1x for 1 minute, then we removed BSA traces with consecutive washes in TBS 1x with 0.01% (v/v) Tween for 5 minutes and then for 15 minutes. Lastly, we moved the blot in a TBS 1x wash for 5 minutes.

The washed blot was incubated in the primary antibody solution composed of TBS 1x, 3% (w/v) BSA, 0.1% (v/v) Tween20, and primary antibody (0.2% for the Anti-Myc and 0.1% for the Anti-FLAG). For immuno-decoration of GLR3.3 we employed a Rabbit anti-Myc primary antibody (AMab9106 antibody from Abcam, <https://www.abcam.com/en-it>), while for the GLR3.7 we used a Mouse anti-FLAG primary antibody (F3165 antibody from Sigma-Aldrich, <https://www.sigmaaldrich.com/>). We incubated the blots at RT for 16 h in constant agitation.

After removing the primary antibody solution, we washed the blot as previously described and proceeded with the secondary antibody tagging. This time we used the same secondary antibody solution composed of TBS 1x, 3% (w/v) BSA, 0.1% (v/v) Tween, and 0.01% (v/v) anti-Mouse Horseradish Peroxidase (A9044 antibody from Sigma-Aldrich, <https://www.sigmaaldrich.com/IT/it>)

or 0.1% (v/v) anti-Rabbit Horseradish Peroxidase (NA934V antibody from Cytiva, <https://www.cytivalifesciences.com/en/us>). We incubated the blot at RT for 1 hour in constant agitation, after which we washed the blot as previously described and started the visualization process.

To visualize the Horseradish Peroxidase secondary antibody binding we incubated our blot into 5 mL of ECL substrate mix (Thermo Fisher, <https://www.thermofisher.com/it/en/home.html>) at RT for 1 minute and visualized the staining at the ChemiDoc chemiluminescent detector (Biorad, <https://www.bio-rad.com/>).

5.5. Histology methods

5.5.1. Vibratome sectioning

Transverse sections of the primary inflorescence for GUS staining were obtained by embedding the sample in 6% (w/v) Agarose using a Vibratome (0.9 mm/s Speed, 1.25 mm Amplitude, 50 μ m Feedrate). Sections were collected in 50 mM Phosphate buffer and kept at 4 °C until staining.

5.5.2. GUS staining

The GUS assay employs a bacterial enzyme (β -Glucuronidase) to catalyse the hydrolysis of the colourless β -Glucoronide substrate into a blue-coloured product. This enzyme can be expressed in plants under specific promoters to verify its tissutal specificity through the substrate hydrolysis and subsequent coloured product production (transcriptional reporter) or fused to a gene of interest to create a chimeric protein, thus also indicating the protein localization (translational reporter). In our case, we employed two translational reporters to identify GLR3.3 and GLR3.7 tissutal localization in adult plants main inflorescence, leaves and stem, and whole seedlings. *pGLR3.3::GLR3.3-GUS* is already published in Nguyen et al., 2018, while *pGLR3.7::GLR3.7-GUS* is presented and characterized in this thesis.

We fixated the samples in 90% (v/v) Acetone at -20 °C for 1 hour, washed them three times in 50 mM Phosphate buffer for 5 minutes, and then incubated in the GUS staining solution (2 mM X-Gluc, 0.1% (v/v) Triton X100, 2 mM $C_6N_6FeK_4$, 2 mM $C_6N_6FeK_3$, 50 mM Phosphate buffer pH 7.0, 10 mM EDTA) first 20 minutes in vacuum (to facilitate penetration of the solution) and then 16 hours at 37 °C. Tissues were then cleared in 70% (v/v) ethanol and mounted in 20% (v/v) glycerol.

5.5.3. *In situ* hybridization

Mature flowers and stems of *Arabidopsis thaliana* were fixed and embedded in 4% paraformaldehyde. Specific GLR3.7 cDNA fragments were amplified by PCR with primers AC404 and AC405 (table 1) and further cloned in pGEM T-easy vector (Promega, <https://ita.promega.com/>). The pGEM::GLR3.7 vector was used as template for the creation of sense and antisense probes by an in vitro transcription performed with SP6 and T7 polymerases. Sections of plant tissue were probed with digoxigenin-labeled antisense RNA-probe as previously described (Coen et al., 1990) and observed in bright field with the SMZ18 stereomicroscope equipped with a 5.9-megapixel CMOS DS-Fi3 Microscope Camera (<https://www.microscope.healthcare.nikon.com/products/cameras/ds-fi3>).

5.6. Microscopy

5.6.1. Confocal laser scanning microscopy

Confocal laser scanning microscopy was employed for localization studies and FLIM analysis of tobacco plants transformed with the pB7FW 2.0 *p35s::GLR3.3-GFP* / pGPTVII *pUBQ10::GLR3.7-mCherry* constructs. Images were acquired on an upright Nikon Eclipse Ti2 microscope with Nikon A1Plus laser scanning module (Nikon, <http://www.nikon.com/>). Localization studies were conducted at high magnification (Objective: CFI Plan Apo 60X Oil Numerical Aperture 1.4) acquiring GFP, excited with the 488 nm laser and collected at 505-550 nm, and mCherry, excited with the 561 nm laser and collected at 576-626 nm. The FLIM analysis were conducted at the same magnification (Objective: CFI Plan Apo 60X Oil N.A. 1.4) plus a 4x digital zoom to better focus on the cell membranes, acquiring only GFP this time using a pulsed 485 nm laser at 40mhz and collecting at 505-550 nm. The microscope was controlled through the NIS-Elements platform (Nikon, <https://www.nis-elements.com/>). For acquisition of fluorescence life time data the PicoQuant SymPhoTime software was employed.

5.6.2. Wide-field fluorescence microscopy

For widefield imaging of seedlings root-tip cells and adult receptacle flower cells we used a Nikon Ti-E inverted microscope (Nikon, <https://www.nis-elements.com/>) equipped with a Prior Lumen 200 PRO fluorescent lamp (Prior Scientific, <https://www.prior.com/>) and a Hamamatsu ORCA-D2 Dual CCD camera (Hamamatsu, <https://www.hamamatsu.com/>). Flower samples were imaged with a CFI

Apo lambda 4X (N.A. 0.2) at 100% fluorescence lamp emission, while seedlings root-tips were acquired with a CFI Plan Apo VC 20X (N.A. 0.75) objective at 20% fluorescence lamp emission. The YC3.6 sensor was excited with a 440 nm filter (436/20 nm) (Chroma Technology, <https://www.chroma.com/>) and collected with a FRET CFP/YFP optical block A11400-03 (ECFP emission 483/32 nm, cpVenus emission 542/27 nm) with a 510 nm dichroic mirror (Chroma Technology, <https://www.chroma.com/>). Seedlings were acquired with exposure of 100-150 ms and 4x4 binning, while inflorescences required 250-500 ms of exposure. Images were acquired every 5 seconds. The microscope was controlled through the NIS-Elements platform (Nikon, <https://www.nis-elements.com/>).

5.6.3. Stereomicroscopy

The Nikon stereomicroscope SMZ18 was employed to screen transformed plants fluorescence and to verify plant responsivity to burning stress before sampling tissues for stress marker gene expression quantification. Acquisition was performed thanks to the Hamamatsu C11440 CMOS camera (Hamamatsu, <https://www.hamamatsu.com/>) and samples were excited with a CoolLED pE-300 ultra at 100% LED emission (CoolLED, <https://www.coolled.com/>) with GFP (at 460 and 405 nm) and RFP cubes (at 561 nm). To acquire the entire plant, we employed a Plan Apo 0.5X objective.

The rehydration experiment was performed on 4-week-old Arabidopsis plants expressing the R-GECO1 calcium sensor. The stimulus (i.e. watering) was induced after 2 minutes from the start of the experiment adding 8 mL of tap water into the base of the 3D-printed chamber. Images were collected only in the RFP channel at 100% LED emission, with an exposure of 1 second, 4x4 camera binning and 1.3X magnification every 5 seconds.

We employed the stereomicroscope also to acquire GUS-stained samples through a CMOS DS-Fi3 microscope camera (Nikon, <http://www.nikon.com/>) and a mercury light source (Intensilight, <http://www.nikon.com/>). The microscope was controlled through the NIS-Elements platform (Nikon, <https://www.nis-elements.com/>).

5.6.4. Florescence Lifetime Imaging (FLIM) analysis

FLIM analysis was performed on *Nicotiana benthamiana* leaves transiently expressing *p35s::GLR3.3-GFP* and *pUBQ10::GLR3.7-mCherry*. After selecting cells expressing either the GLR3.3-GFP or the combination of GLR3.3-GFP and GLR3.7-mCherry, we acquired the GFP photon decay by exciting it with a pulsed 485 nm laser at 40 mhz. As reference we used 1 mM Coumarin6

diluted in EtOH 100% (known photon lifetime around 2.5 ns). We collected the image through the SymPhoTime 64 software (PicoQuant, <https://www.picoquant.com/>) reaching at least 500 photon counts per pixel and recorded photon decay in binary files. The files were then analysed by the FLIM STUDIO Software (FLIM LABS, <https://www.flimlabs.com/about-us/>) through a phasor plot analysis.

5.7. Image post-processing

5.7.1. Genetically encoded indicators analysis

All images were analysed using FIJI-software (<https://www.fiji.sc/>). For each sensor the emission intensity was quantified as mean grey value intensity in a given Region of Interest (ROI) (indicated in the figures). We subtracted from the ROI intensity the background as mean grey value intensity of an area outside the sample from each acquired channel separately. Output was then analysed in Excel (<https://www.microsoft.com>) as following.

Fluorescence values from the intensimetric sensors (e.g. R-GECO1) were normalized on the mean grey value intensity value representative of the pre-stimulus condition (F_0) as $\Delta F/F_0$ ($\Delta F = F_t - F_0$ in which ΔF is the variation of F_t , the grey value of a given timepoint, compared to the pre-stimulus condition F_0).

Data on root-tip cells and receptacle flower cells obtained with the ratiometric YC3.6 was first processed by obtaining a Ratiometric trace (R) between the YFP (cpVenus) and CFP mean grey value (subtracted from the background). Then the ratio was normalized on the pre-stimulus condition (R_0) as $\Delta R/R_0$ ($\Delta R = R_t - R_0$ in which ΔR is the variation of R_t , the grey value of a given timepoint, compared to the pre-stimulus condition R_0).

5.7.2. Phasor plot analysis of Fluorescence lifetime

The phasor plot is graphical representation of the lifetime distribution within the sample. It is commonly used in fluorescence lifetime imaging microscopy (FLIM) to visualize the decay of fluorescence emission from a molecule over time. The interpretation of the FLIM phasor plot is straightforward and the combination of FLIM and phasors enables the researchers to easily separate different lifetime populations within the same FLIM image. The main constraint of this approach is that the instrument response widely influences the precision of the analysis, so we require a reference analysis to probe the instrument precision and response. The output of each FLIM dataset is a 3D

matrix, with X and Y as coordinates of each pixel in the image (Fig 10.a and 10.d) and the third coordinate is T, the Laser Period, indicating the fluorescence intensity decay profile behind each single pixel (Fig 10.b and 10.e).

The projection of the reference, a standard solution of a known fluorophore, is collapsed into a single coordinate (Fig 10.b) as each pixel should represent the decay of copies of the same molecule in a known liquid media. This in turn produces a precise decay profile of the reference in our experimental conditions. Being a known fluorophore, the reference spectra is used to extrapolate, through a phasor transformation function (PTF), the parameters of the Instrument Response (the phase component ϕ_{IR} and the amplitude component m_{IR}) that modify the acquired decay from the expected one (Fig 10.c). The phase component (ϕ_{IR}) indicates the degree to which the instrument's output signal lags behind or leads the reference phasor. A small phase angle indicates that the instrument is accurate and precise while a large phase angle indicates that the instrument is probably inaccurate. The magnitude component (m_{IR}) indicates the ratio of the instrument's output signal to the reference phasor. A large magnitude component indicates that the instrument is sensitive, while a small magnitude component indicates that the instrument is insensitive.

When we analyse our dataset, we extrapolate the amplitude and phase of each pixel's Laser Period (Fig 11.e) and correct them with the Instrument Response obtained from the reference. The output of this process are two coordinates, g and s, respectively the real part (amplitude) and imaginary part (phase) of the corrected PTF. These corrected data can fit into the Phasor plot (Fig 10.f).

The real part of the complex number is plotted on the x-axis (g), and the imaginary part is plotted on the y-axis (s). The length of the phasor represents the amplitude of the signal (m), and the angle of the phasor represents the phase of the signal (ϕ). Onto the plot lies a curve describing all populations characterized by a mono-exponential decay (Fig 10.g), while a slower decay is characterized by a population localizing in the right side of the plot (Fig 10.h).

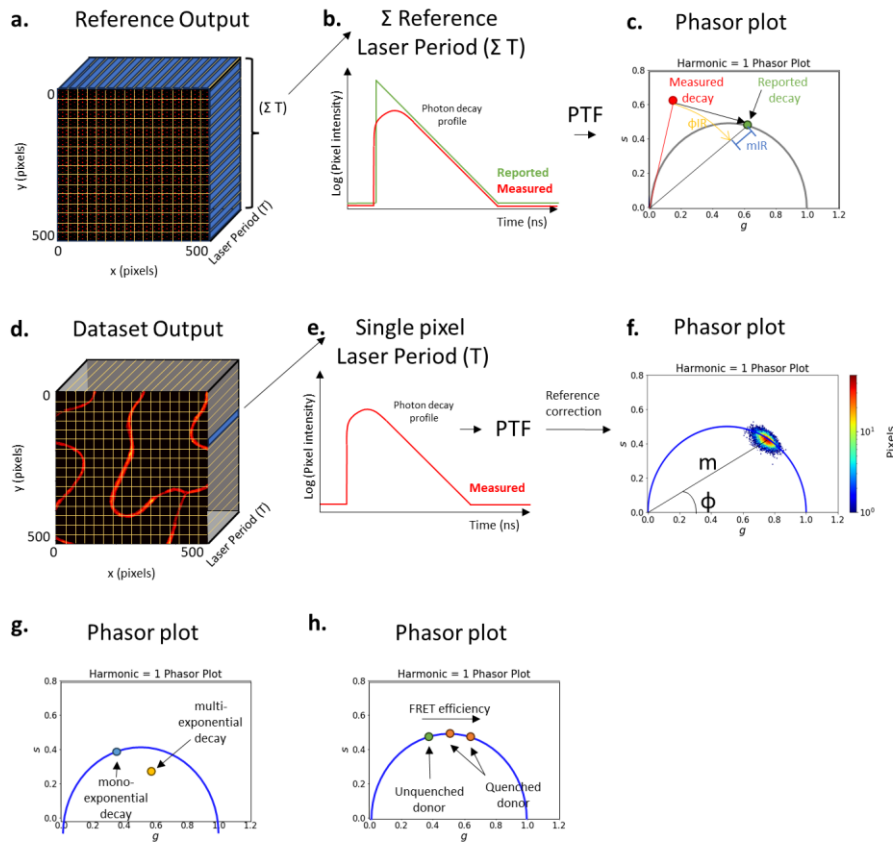


Figure 10 – Graphical representation of a phasor plot analysis. (a.) A reference sample is used to collapse all pixels Laser Period (ΣT) into a trace describing the decay of the reference fluorophore (b.). This information is used, through a phasor transformation function (PTF), to extrapolate the phase (ϕ_{IR}) and magnitude (m_{IR}) component of the instrument response (c.). (d.) From a sample dataset, a single pixel laser period (e.) can be extracted and corrected thanks to the Instrument Response previously calculated. (f.) The corrected pixel decay is fitted into a Phasor Plot and a script can recognize and define a population of pixels that belong to the same fluorophore. (g.) visual representation of the different position occupied by the centroid of a mono and multi-exponential population of fluorophores. (h.) visual representation of the different position occupied by the same mono-exponential population of a donor fluorophore subject to FRET.

5.8. Statistical analysis

All traces and histograms are averages representative of $n \geq 3$ experiments and reported with the relative standard deviation (SD). P-values were calculated with a two-sided unpaired Student's t-test with 95% confidence interval for 1 vs 1 comparison of two populations, while for multiple comparisons of more than 2 populations we used a two-sided unpaired One-Way ANOVA test with 95% confidence interval and post-hoc Tukey test. Data was plotted in GraphPad Prism (GraphPad, <https://www.graphpad.com/>).

5.9. Additive manufacturing

All growing custom supports and imaging chambers for Arabidopsis have been produced by means of three-dimensional (3D) printing additive manufacturing.

Original 3D models have been designed in Autodesk Fusion 360 (Autodesk, <https://www.autodesk.com/>) and exported as Standard Triangulation Language (STL) files. The Files have been loaded and processed in the open source Slic3r software (Slic3r, <https://slic3r.org/>) and converted into the 3D printing programming language gcode.

The code was printed at 100% infill, 0.3 mm layers by the Anycubic Vyper 3D printer (Anycubic, <https://www.anycubic.com/>) equipped with a 0.8 mm nozzle. Models were realized in Polylactic acid (PLA) (3D Store Monza, <https://www.3dstoremonza.it/>) at 200 °C nozzle temperature and 60 °C heated bed temperature.

5.10 List of primers used in this work

Table 1 – Primers sequence for molecular biology applications

Name	Primer sequence	Direction	Locus	Purpose
AC901	CACCAGATGCGTTGGGGAT	For	At2G17260	Realtime analysis of ATGLR3.1 expression
AC902	CTGACTGAGACCCATGCGGG	Rev		
AC903	CACTCGGTCGTGCTGTTCTG	For	At4G35290	Realtime analysis of ATGLR3.2 expression
AC904	AACCCAACTCGTCCACTGCT	Rev		
AC402	ACGTTGGGAAAAAGCGGAAA	For	At1G42540.1	Realtime analysis of ATGLR3.3 expression
AC403	ACCATACGCGTTCCGAGGAT	Rev		
AC905	AGGTACAGCTCCACGCACAG	For	At1G05200.1	Realtime analysis of ATGLR3.4 expression
AC906	AACACCCAACCACGAGGAGG	Rev		
AC907	ACGCGCTGTATGCTTACGAT	For	At2G32390	Realtime analysis of ATGLR3.5 expression
AC908	TGACCTGCAAGAACCTCTCC	Rev		
AC416	GGGAAAGGCAGCTTGAAGAT	For	At3G51480	Realtime analysis of ATGLR3.6 expression
AC417	TGAGGTCTCTTTGGTGCAAC	Rev		
AC404	TACCAGGCTGGGACGTTTAC	For	At2G32400.1	Realtime analysis of ATGLR3.7 expression and <i>in situ</i> hybridization
AC405	TCCTCGGTCGAGTCAAGTGG	Rev		
AC348	AGGGAAACGAAGACAGCAAG	For	At5G44340	Realtime analysis of TUB4 expression
AC349	GCTCGCTAATCCTACCTTTGG	Rev		
AC597	CATGGCGGCCGCATGGGACTGGGCATTGACCCAT	For	At2G32400.1	Primers for GLR3.7 coding sequence cloning in pESC-LEU vector
AC598	CATGGCGGCCGCTCCATTTTCGTGGTACCTCAGTA	Rev		
AC595	ATACGACTCACTATAGGGCCATGAAGCAACTCTGGACTTTC	For	At1G42540.1	Primers for GLR3.3 coding sequence cloning in pESC-LEU vector
AC596	TCCATGTCGACGCCCCGGCCTCCGTCTAATGGATTACC	Rev		
AC606	CAGTGGATCCATGTTACGTCCTGTAGAAAC	For	Ec3.2.1.31	Primers for GUS cloning in pGreen0029 vector
AC607	CATGGCGGCCGCTCATTGTTGCCTCCCTGCT	Rev		
AC433	CATGGAATTCTATTATGTAATAGTTTTTGCCGC	For	At2G32400.1	Primer for GLR3.7 cloning in pGreen0029 vector
AC609	CATGCCCGGGTCCATTTCGTGGTACCTCAGTA	Rev		

6. Results

6.1. The analysis of the expression patterns of GLR3.3 and GLR3.7 shows an overlap at different developmental stages

We reasoned that to support the possible formation of a heteromeric GLR3.3 - GLR3.7 channel there is the need to show that these two proteins are expressed in the same cell type or tissue. Thus, to study the expression pattern of GLR3.7 and if it correlates to the one of GLR3.3 we chose to use translational reporters, made by the fusion of the full-length genomic sequences (promoter, exons, and introns) of both GLR3.3 and GLR3.7, to the coding sequence of the *uidA* gene, coding for the β -Glucuronidase enzyme (Jefferson et al., 1987). By using a translational reporter, we can appreciate both the expression pattern and the stability of the protein.

We thus employed the already published *pGLR3.3::gGLR3.3-GUS* (Nguyen et al., 2018), and a newly generated *pGLR3.7::gGLR3.7-GUS* (see Materials and Methods for the design of the construct and the plant transformation) *Arabidopsis* translational GUS reporter lines. Plants at different developmental stages were then analysed to detect the activity of the GUS enzyme.

To what concern the GLR3.3-GUS line we identified a clear GUS activity in different tissues of 7-day-old seedlings, like the cotyledons (Fig 11.c) and root vasculature (Fig 11.d) and the very root tip (Fig 11.e), supporting the role of GLR3.3 on AA perception in this tissue (Alfieri et al., 2020; Grenzi et al., 2023). We then analysed the GUS activity in adult GLR3.3-GUS plants. The GUS activity was detected in the inflorescence stem (Fig 11.a), limited to the phloem tissues as revealed by the GUS analysis on transverse sections (Fig 11.b), in the flowers where the blue stain was present in the receptacle cells, in the sexual organs like stamens and pistil. The GLR3.3 expression in the stem phloem and flower receptacle cells support its involvement in the long-distance $[Ca^{2+}]_{cyt}$ increases observed in these tissues in response to the leaf burning (Grenzi et al., 2023).

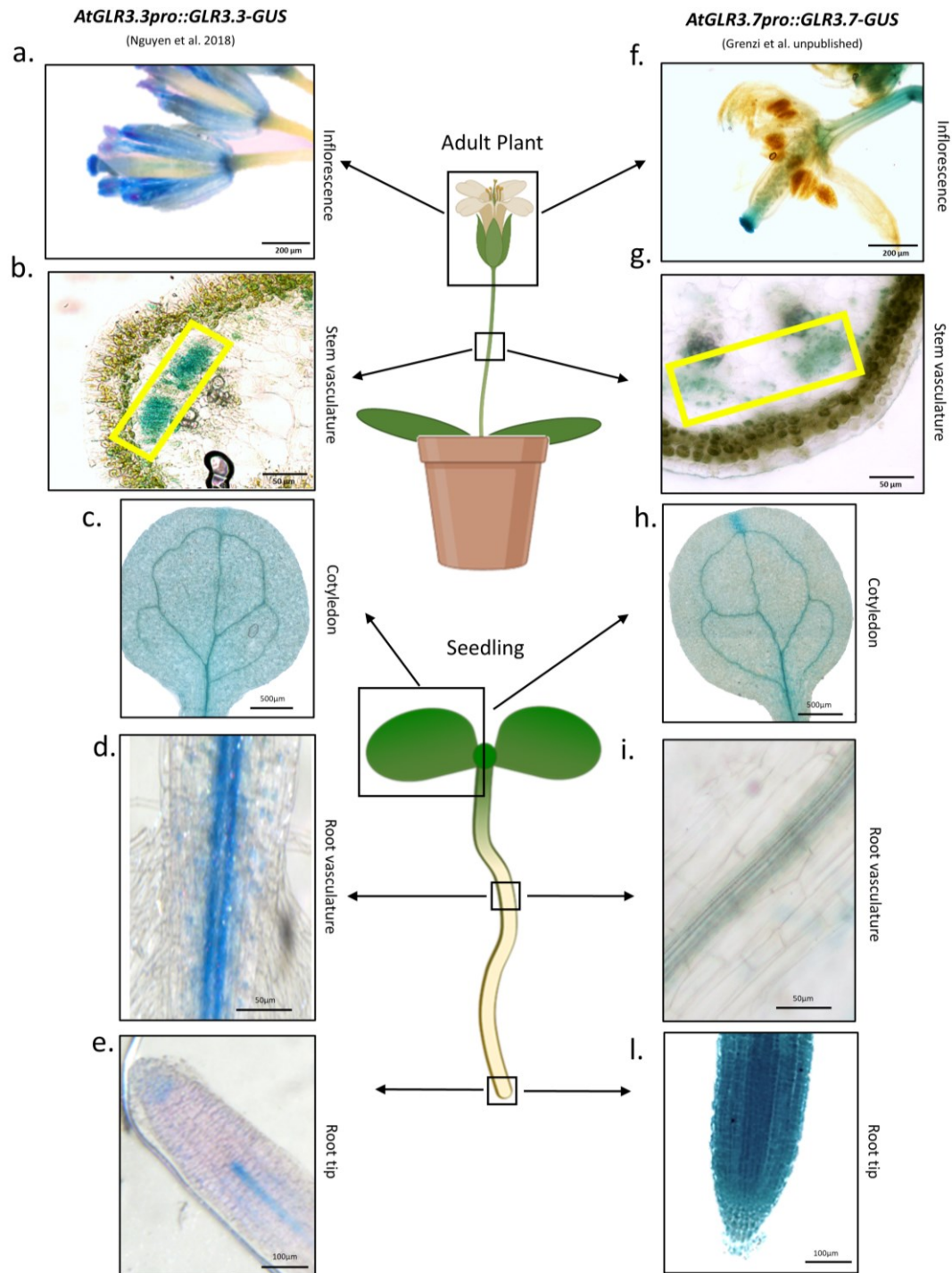


Figure 11 – Expression pattern of GLR3.3 and GLR3.7 in *Arabidopsis*. Representative GUS staining for pGLR3.3::GLR3.3-GUS (a-e) and pGLR3.7::GLR3.7-GUS (f-l) transcriptional reporter in multiple tissues. In order: a,f, adult plant inflorescence; b,g, transverse section of adult plant main inflorescence stem vascular bundles; c,h, seedling cotyledon; d,i, seedling main root; e,l, seedling root tip.

The GUS analyses were then repeated with the GLR3.7-GUS plants at different developmental stages. In 7-day-old seedlings the GUS staining was intense in the root tip meristem (Fig 11.l), faint in the root vasculature (Fig 11.i) and clearly visible in the vasculature of the cotyledon (Fig 11.h) with similar cell populations expressing GLR3.3. In flowers, GLR3.7 shows an expression pattern limited to vegetative organs, from the flower peduncle to the receptacle, but seemingly absent in sexual

organs (Fig 11.f). To better define in which type of vascular tissue the GLR3.7 was present, we prepared transverse section of the stem, which revealed the presence of GUS staining in both phloem and xylem (Fig. 11.g). This data agrees with *in situ* hybridization analyses previously performed in the laboratory showing that *GLR3.7* mRNA is present in both vascular tissues (Resentini et al., unpublished, Fig. 12).

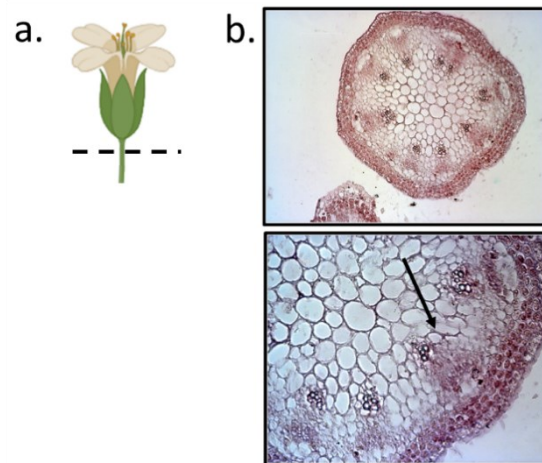


Figure 12 - *AtGLR3.7* *in situ* hybridization, Resentini et al., unpublished. (a.) transverse section of adult *Arabidopsis* plants inflorescence stem used for *in situ* hybridization. (b.) *in situ* hybridization of *AtGLR3.7*. The arrow points at the hybridized vascular bundle.

Overall, by performing a side-by-side comparison of the GUS staining results, obtained with the two lines, we can assess that GLR3.3 and GLR3.7 expression patterns partially overlap, in the stem vasculature and seedling root (Table 2).

Table 2 – Overview of the expression pattern of pGLR3.3::*GLR3.3*-GUS and pGLR3.7::*GLR3.7*-GUS translational reporters

	pGLR3.3:: <i>GLR3.3</i> -GUS	pGLR3.7:: <i>GLR3.7</i> -GUS
Floral tissues	Stamen, pistil, petals, receptacle	Pistil, petals, floral pedicel vasculature
Main inflorescence stem	Phloematic vascular bundles	Phloematic and xylematic vascular bundles
Seedling cotyledon	Vascular bundles	Vascular bundles
Seedling main root axis	Vascular bundles	Faint in vascular bundles
Seedling main root tip	Vascular bundles, root tip	Whole root tip

6.2. GLR3.3 and GLR3.7 show close proximity in leaf epidermal cells of *Nicotiana benthamiana*

GLR3.3 was reported to localize at the plasma membrane (Bellandi et al., 2023; Vincill et al., 2013) as well as in the endoplasmic reticulum (ER) of phloematic cells (Nguyen et al., 2018). Having shown that GLR3.3 and GLR3.7 can be co-expressed in the same tissues (i.e. phloem), our hypothesis is that GLR3.7 could exert its regulatory role on GLR3.3 through a direct interaction, possibly by forming a hetero-tetrameric channel. To test this hypothesis, we wanted to further investigate the subcellular localization of the two GLRs by tagging each isoform with a different fluorophore. We, thus, generated two chimeric constructs with the *GLR3.3* coding sequence fused to *GFP* and the *GLR3.7* coding sequence fused to the *mCherry*, placed under control of the CaMV35 and pUBQ10 promoters, respectively (*p35s::GLR3.3-GFP* and *pUBQ10::GLR3.7-mCherry*).

We transiently transformed *Nicotiana benthamiana* leaf epidermal cells by means of *Agrobacterium* mediated infiltration (Waadt & Kudla, 2008). *N. benthamiana* was chosen as model organism for this assay as it conveniently allows for a fast and reliable approach in alternative to a stable transformation in *Arabidopsis thaliana* (Waadt & Kudla, 2008). We performed two different experiments: we transformed leaf cells with i) the *GLR3.3-GFP* construct alone (Fig 13.a and 13.b), or in combination with ii) the *GLR3.7-mCherry* (Fig 13.c and 13.d). After 4 days from the infiltration, we analysed the transformed epidermal cells by means of fluorescence laser scanning confocal. Cells expressing the *GLR3.3-GFP* showed a fluorescence signal that was localized at the periphery of the cell which might suggest a plasma membrane localization (Fig 13.a and 13.b). However, by performing a higher magnification analysis, we clearly detected a non-uniform distribution of the fluorescence that also decorated intracellular membranes, possibly the ER (Fig 13.b). Leaf cells co-expressing both *GLR3.3-GFP* and *GLR3.7-mCherry* (Fig 13.c and 13.d) showed a clear colocalization of the two fluorescence emissions, as demonstrated by the plot profile, suggesting that both proteins reside in the same membranes.

Whereas this result suggests a colocalization of the two channels it cannot provide information regarding their interaction. Thus, with the same material, we carried out a Fluorescence Lifetime Imaging Microscopy-Fluorescence Resonance Energy Transfer (FLIM-FRET) analysis to test if the two channel proteins are in close-proximity, a requisite necessary to support their interaction.

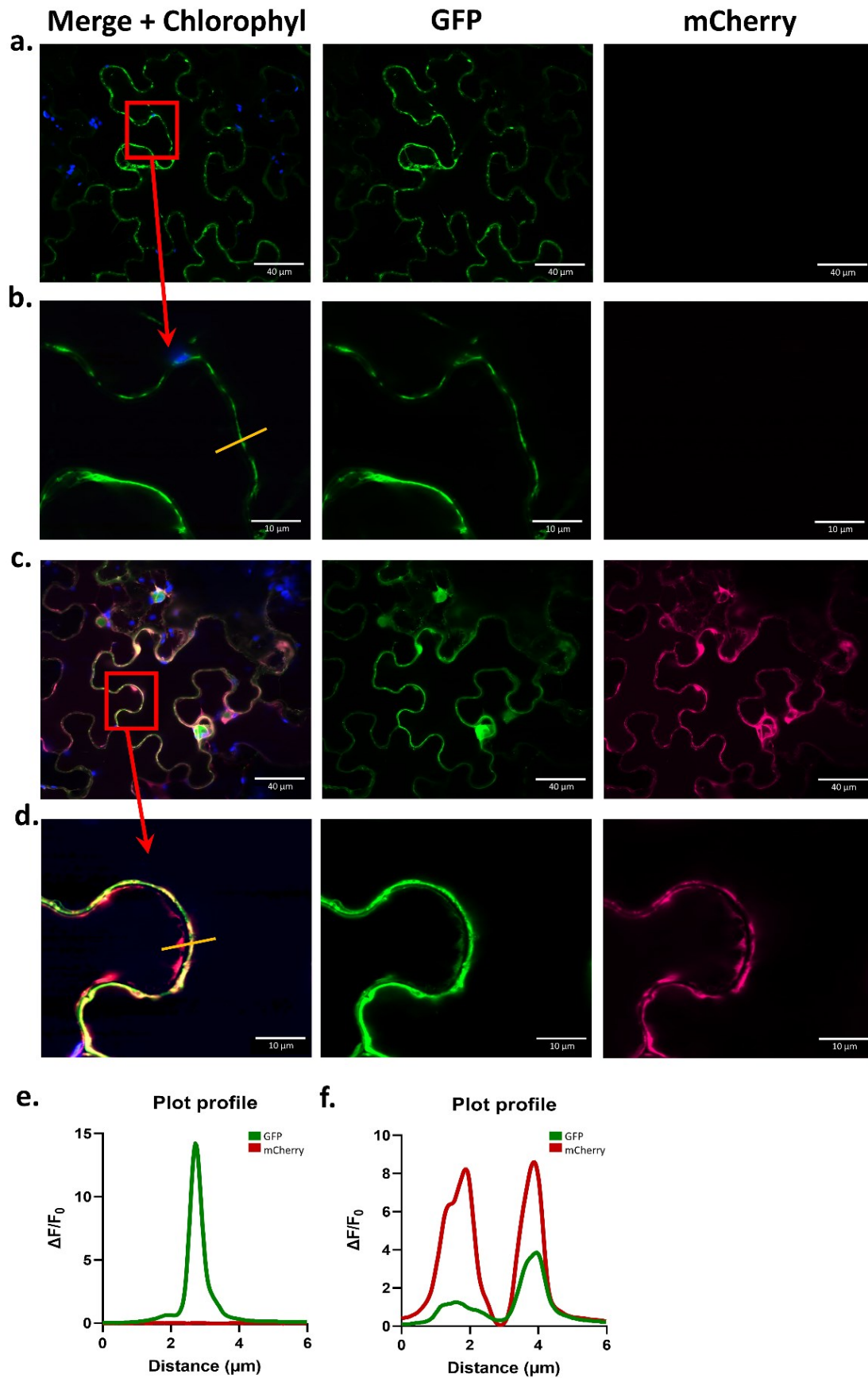


Figure 13 – Transient expression of GLR3.3-GFP and GLR3.7-mCherry in *Nicotiana benthamiana* leaf epidermal cells. In order from left to right we have representative images of the merged acquisition and single GFP and mCherry emission channels (green and red

respectively). In merged images, the Chlorophyll B emission is represented in blue. (a.) Subcellular localization of GLR3.3-GFP expressing epidermal cells at 60X magnification with 1X digital zoom. (b.) Subcellular localization of GLR3.3-GFP expressing epidermal cells at 60X magnification with 4X digital zoom. In yellow highlighted the region used for the subsequent plot profile analysis (e.). (c.) Subcellular localization of epidermal cells co-expressing GLR3.3-GFP and GLR3.7-mCherry at 60X magnification with 1X digital zoom. (d.) Subcellular localization of epidermal cells co-expressing GLR3.3-GFP and GLR3.7-mCherry at 60X magnification with 4X digital zoom. In yellow highlighted the region used for the subsequent plot profile analysis (f.).

In FLIM-FRET, the technique relies on the relative positions of two fluorescent molecules with partially overlapping spectra, which is a crucial requirement for FRET to occur. When these fluorophores are in close proximity (typically within 1-10 nanometers), FRET can take place (Krishnan et al., 2001). Specifically, in this technique the donor fluorophore, must feature an emission spectrum that, at least partially, overlaps with the excitation spectrum of a second fluorophore, called acceptor fluorophore. In this way if the donor fluorophore (in our case, the GFP), is in an excited state, the excited electrons can decay emitting photons, that can be adsorbed by the acceptor fluorophore (in our case the mCherry), through a non-radiative transfer of energy. Importantly, this FRET event not only excites the acceptor fluorophore, but also increase the decay probability and speed of the donor emitted photons, which is something that can be precisely quantified (Krishnan et al., 2001).

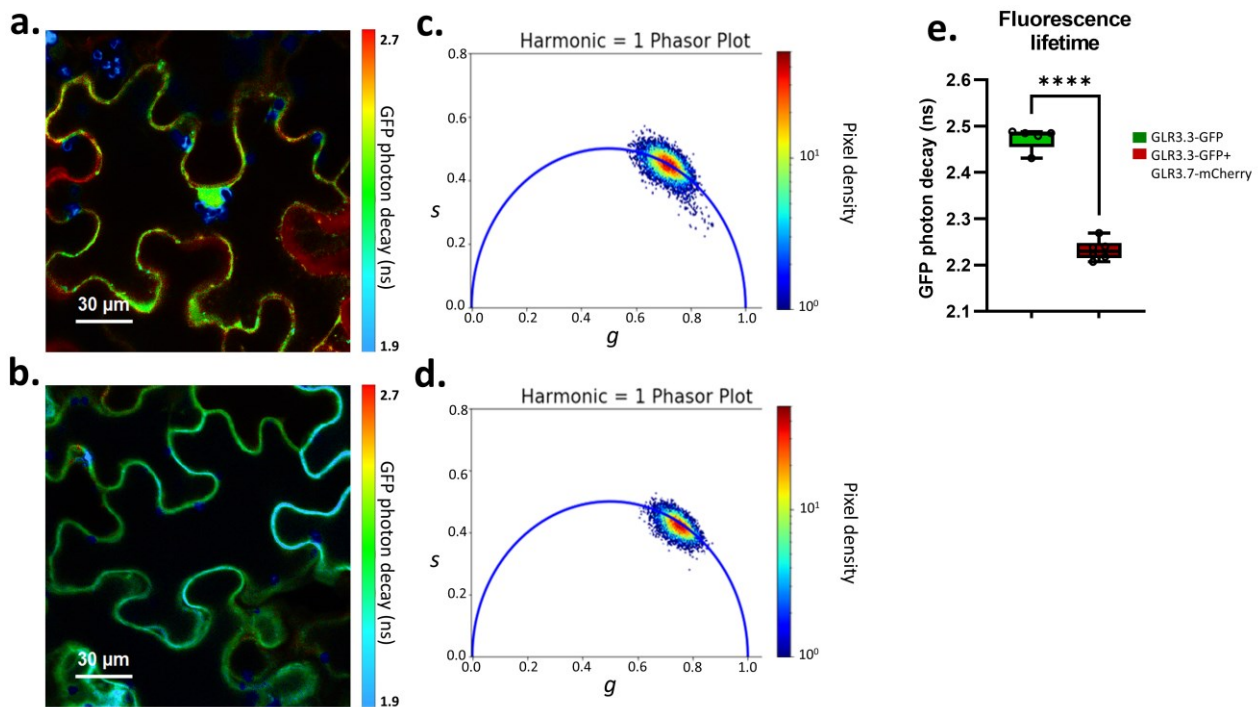


Figure 14 – FLIM-FRET analysis of GLR3.3-GFP and GLR3.7-mCherry. Visual representation of the GFP photon decay of plants expressing only GLR3.3-GFP (a.) and co-expressing GLR3.3-GFP and GLR3.7-mCherry (c.). A pseudo-coloured Physics LUT scale is used to visually represent the GFP photon decay, with colder colours indicating a faster decay. Phasor plot of the GFP photon decay

distribution of plants expressing only GLR3.3-GFP (b.) and co-expressing GLR3.3-GFP and GLR3.7-mCherry (d.). (e.) Mean fluorescence lifetime of emitted GFP photons. $n > 4$. Error bars = SD. * $P < 0.0001$ (Student *t* test).

As stated above, our donor fluorophore is a GFP, tagging the GLR3.3 protein, while as an acceptor we use the mCherry fused to GLR3.7. To precisely quantify the photon decay time of GFP we performed a real-time FLIM analysis using a phasor-plot approach (FLIM STUDIO Software, <https://www.flimlabs.com/about-us/>, see Materials and Methods for a detailed description of the acquisition setup and phasor analysis). The phasor approach allowed us to define if the GFP photon decay time was a single exponential lifetime decay in both conditions, with or without GLR3.7-mCherry (Fig 14.b and 14.d) and allowed us to obtain the values of the decay times in the two tested conditions. Based on this analysis we observed that when expressed alone, the GFP bound to GLR3.3 shows an averaged photon decay time of 2.474 ± 0.021 ns, but when co-expressed with the GLR3.7-mCherry the averaged photon decay time decrease to 2.232 ± 0.020 ns (Fig 14.e). The decreased decay time is indicative of the FRET occurrence, thus, supporting the evidence that when expressed in *Nicotiana benthamiana* leaf epidermal cells GLR3.3 and GLR3.7 not only colocalize but interact.

6.3. Co-IP assay confirms direct interaction between GLR3.3 and GLR3.7 in yeast

In different isoforms of animals orthologs iGluRs, it has been widely reported the ability to assemble into functional heterotetramers (Karakas & Furukawa, 2014; Traynelis et al., 2010). This possibility provides the channel with an added layer of regulatory plasticity as every isoform can modulate the channel gating and permeability (Traynelis et al., 2010).

As I previously shown, in Arabidopsis plants GLR3.3 and GLR3.7 can be present in the same tissues, and in tobacco localize in the same membranes where they potentially interact (Fig 13.b and 13.c). These results further support our hypothesis for the formation of a complex between GLR3.3 and GLR3.7. In order to further strengthen this hypothesis, aside from the FLIM assay which proves that the two channels are in a close proximity, we decided to perform a Co-Immunoprecipitation assay. We selected as a model organism the yeast *Saccharomyces cerevisiae* whose genome does not have GLR orthologous genes (Chiu et al., 1999).

In particular, we used the G19 strain that is a sodium (Na^+)-extruding ATPase deficient mutant of *S. cerevisiae* (Horie et al., 2001; Quintero et al., 1996). This strain shows a strong sensitivity to external Na^+ when present at high concentration, thus it can be used to test the permeability of channels or transporters to Na^+ (Horie et al., 2001). Importantly, GLR3.3 has shown Na^+ permeability in COS-7 mammalian cells (Wudick et al., 2018). To perform this analysis, we generated a series of constructs

based on the *pESC-LEU* yeast epitope tagging vector (Agilent technologies, <https://www.agilent.com/>), an inducible double cassette vector with whom one or two cloned genes can be introduced into a yeast host strain under the control of a repressible promoter. We thus generated the following vectors: *pESC-LEU-pGAL1::GLR3.3-cMyc- pGAL10::GLR3.7-FLAG*; *pESC-LEU-pGAL1::GLR3.3-cMyc- pGAL10-FLAG*; *pESC-LEU-pGAL1- cMyc - pGAL10::GLR3.7-FLAG* (see Materials and Methods for the construct design) and transformed the G19 yeast cells. As a control we also transformed the yeast cells with the *pESC-LEU* empty vector (Fig 15.a).

The four yeast strains, harbouring the different constructs were grown in SC-LEU media, with Galactose to induce gene expression. When reached an OD₆₀₀ of around 1.0, cells were collected and the total cell lysate extracted, to perform the immunoprecipitation assay. Total cell lysates were loaded onto a column immunodecorated for the cMyc epitope tagging the GLR3.3. Specifically, we focused onto four fractions: the Loaded, representing the total cell lysate, the Flow Through, containing the cell lysate flowing passively through the column, the Wash, containing the unbound proteins eluted with a mild washing buffer, and the Eluted fraction, containing the proteins directly bound onto the column eluted with denaturing elution buffer. The proteins contained in the four fractions were then separated with an SDS page and transferred onto a nitrocellulose blot then used for immune-decoration against the two tagging epitopes.

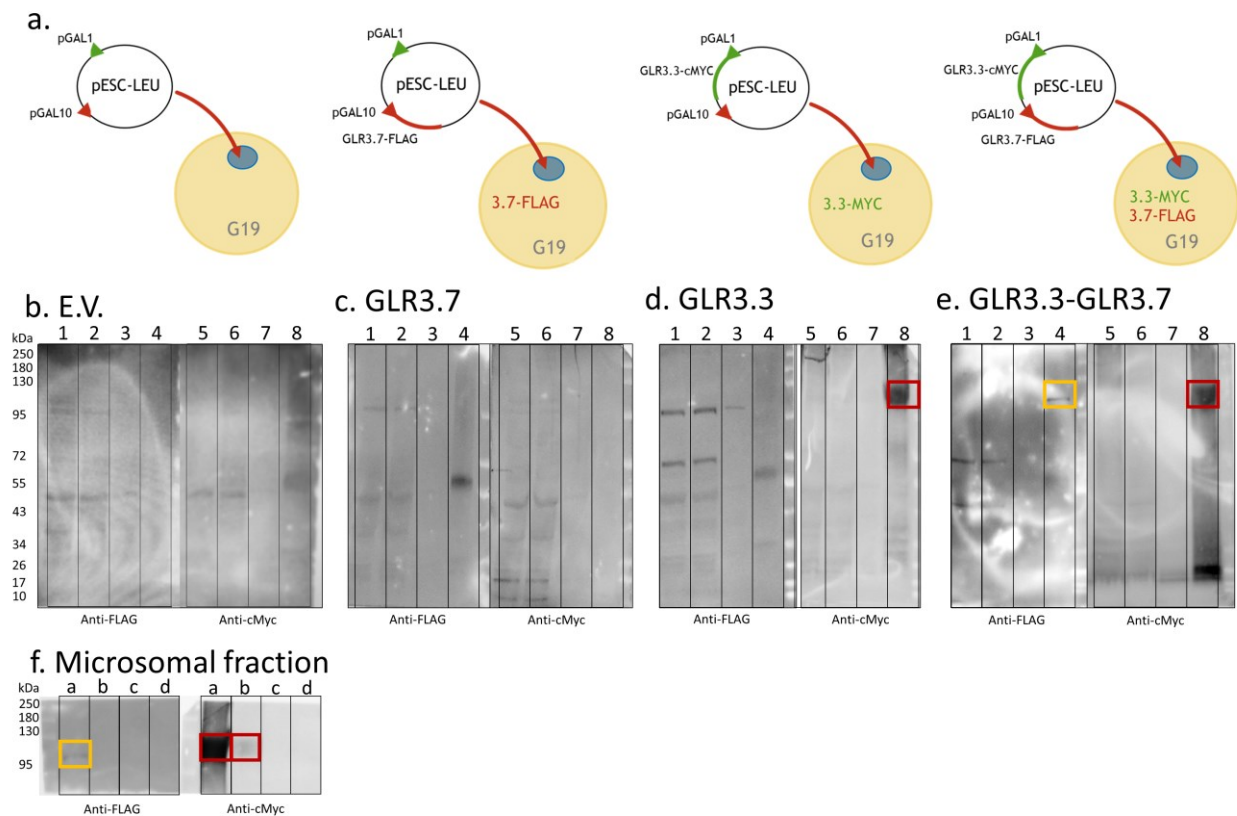


Figure 15 – Co-Immunoprecipitation of GLR3.3 and GLR3.7 in the heterologous system *Saccharomyces cerevisiae*. (a.) experimental setup of the four expression vectors used for the Co-IP assay. From left to right: Empty Vector, GLR3.7-FLAG, GLR3.3-cMyc, GLR3.3-cMyc+GLR3.7-FLAG. (b.) Western Blot for the Empty Vector strain. (c.) Western Blot for the strain expressing GLR3.7-FLAG. (d.) Western Blot for the strain expressing GLR3.3-cMyc. (e.) Western Blot for the strain expressing both GLR3.3-cMyc and GLR3.7-FLAG. In figures b to e lane 1 and 5 refers to the Loaded fraction, lanes 2 and 6 to the Flow Through fraction, lanes 3-4 to the Wash fraction, and lanes 4 and 8 to the Elution fraction. (f.) Western Blot for the microsomal fraction of the four aforementioned strains, (f.a) GLR3.3-cMyc and GLR3.7-FLAG expressing strain, (f.b) GLR3.3-cMyc expressing strain, (f.c) GLR3.7-FLAG expressing strain, (f.d) Empty Vector expressing strain. All western blots have been immunodecorated for the c-Myc epitope (Anti-cMyc) and FLAG epitope (Anti-FLAG). In red are the bands with molecular weight compatible to GLR3.3 and in yellow the bands compatible to GLR3.7.

As expected, the result of this procedure shows a clear and specific Anti-cMyc labelling compatible with the molecular weight of GLR3.3 in the Eluted fraction in both lines, expressing only the GLR3.3 (Fig 15.d) or the Double Vector harbouring both GLR3.3 and GLR3.7 (Fig 15.e), but not in the Empty Vector (Fig 15.b) or single vector expressing only GLR3.7 (Fig 15.c). Furthermore, decoration against the FLAG tag, shows an interesting result. We can appreciate a band compatible with GLR3.7 molecular weight in the Eluted fraction of the line expressing both GLR3.3 and GLR3.7 (Fig 15.e), but not in the line expressing only GLR3.7 (Fig 15.c). This result indicates a retainment of GLR3.7 in the immunoprecipitation column immune-decorated for the cMyc epitope only if expressed in parallel of GLR3.3, a result expected if GLR3.3 itself binds the GLR3.7 crosslinking it to the separation column.

The absence of the proteins of interest in the Loaded fraction of all our sample is possibly due to a low amount of the channel proteins in yeast cells, as just by extracting the microsomal fraction of our transformed yeast strains, enriching insoluble proteins, we were able to verify the accumulation of GLR3.3 in the single line expressing it (Fig 15.f.b), however we were not able to obtain a sufficient accumulation of GLR3.7 in the line expressing the isoform alone (Fig 15.f.c).

6.4. GLR3.7 revert GLR3.3-dependent growth defects in yeast cells

When we performed the Co-Immunoprecipitation assay, we noticed a peculiar behaviour of our yeast strains. When growing our cultures and inducing the expression of the constructs we always observed a reduced growth of yeast cells expressing *GLR3.3* alone. Since this effect was repeatedly observable in multiple replicates, we decided to delve into it better investigating *GLR3.3* effects on yeast growth. We employed the same G19 yeast strain expressing the different combinations of *GLR3.3* and *GLR3.7* in the *pESC-LEU* vector and characterized the growth through a complementation assay. This technique involves cultivating liquid cultures of different yeast strains under non-inducing conditions until they reach a similar growth stage, as determined by measuring the OD₆₀₀ value of the culture. Upon reaching this stage, the yeast cultures are spotted onto solid media at various starting

concentrations (in this case, cells were quantified based on their OD₆₀₀ value and the chosen starting concentrations were 0.1, 0.01, and 0.001 A) under both non-inducing and inducing conditions. The growth efficiency is then assessed based on the area occupied by the culture spots after a predetermined period.

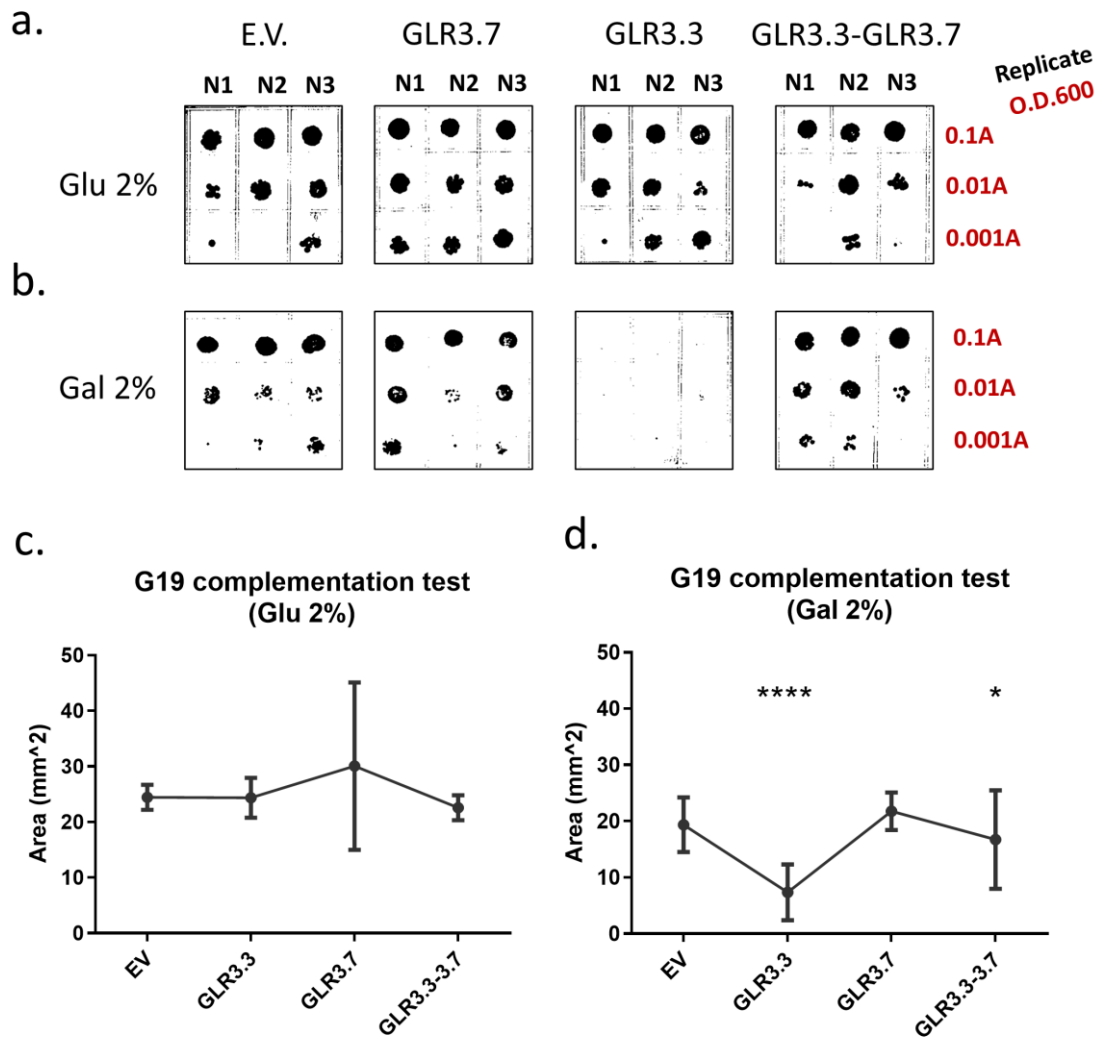


Figure 16 – Complementation assay of GLR3.3 and GLR3.7 in *S. cerevisiae*. (a.) Enhanced contrast images of yeast colonies grown in non-inducing (Glu 2%, media enriched with 2% w/v Glucose) conditions expressing the four expression vectors at three starting concentrations (0.1 A, 0.01 A, 0.001 A). (b.) Enhanced contrast images of yeast colonies grown in inducing (Gal 2%, media enriched with 2% w/v Galactose) conditions expressing the four expression vectors at three starting concentrations (0.1 A, 0.01 A, 0.001 A). (c.) Quantification of colonies total area in non-inducing conditions at starting concentration 0.1 A. (d.) Quantification of colonies total area in inducing conditions at starting concentration 0.1 A. *n* = 9. Error bars = SD. * *P* < 0.05, **** *P* < 0.0001 (One-way ANOVA test).

By following the yeast growth on non-inducing conditions (Glucose enriched solid growth media), all strains exhibit similar growth, indicating no interference of the vector itself onto yeast propagation (Fig 16.a). Instead, when the yeast cells were spotted onto the Galactose enriched media, the result

was different. Yeast cells expressing the GLR3.3 alone grew visibly less than the control cells transformed with the Empty Vector (Fig 16.b). Interestingly, the sole expression of *GLR3.7* did not affect the growth of yeast and even more important its presence seems to revert the effects of *GLR3.3* expression on the area occupied by the colonies (Fig. 16.d). This latter result supports the possibility that GLR3.7 does not only interact with GLR3.3 but also affects its activity in yeast. Certainly, this piece of evidence agrees with the data obtained in Arabidopsis mutant plants, where the lack of *GLR3.7* leads to exacerbated Ca^{2+} responses to exogenous administration of AA or mechanical injury (see Introduction).

6.5. GLRs from clade 3 do not show altered expression in the *glr3.7* mutant backgrounds

Based on the previous findings, we can summarize that GLR3.7 seems to be a negative regulator of GLR3.3 activity, in response to different external stimuli. While this seems to be also confirmed by the molecular interaction of the two isoforms, we hypothesized that other GLRs in clade 3, including GLR3.3, might compensate for the loss of a single isoform, such as in the *glr3.7* mutant background, and explain the increased sensitivity to stressors. Therefore, we wanted to assess if the ablation of GLR3.7 could influence the expression level of Clade 3 GLR genes. For this purpose, we quantified the expression level of GLR clade 3 isoforms in Arabidopsis seedlings, by means of quantitative Real-Time qPCR.

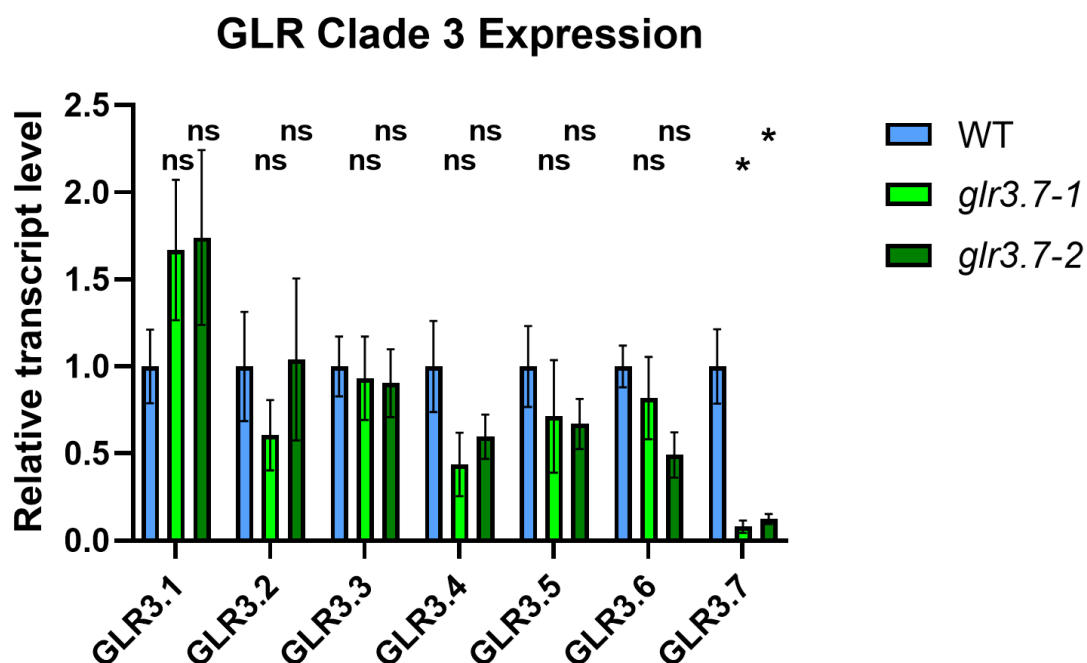


Figure 17 – GLR Clade 3 expression level in GLR3.7 mutant backgrounds. Relative transcript level calculated on the reference gene *Tubulin 4* of GLR isoforms of clade 3 in WT, *glr3.7-1*, and *glr3.7-2* whole seedlings backgrounds. Error bars = SD. * $P < 0.05$ (One-way ANOVA test).

We sampled 7-day-old seedlings from WT and two mutant alleles, *glr3.7-1* and *glr3.7-2*. We then characterized the normalized expression level of *GLR3.1* (*At2G17260*), *GLR3.2* (*At4G35290*), *GLR3.3* (*At1G42540.1*), *GLR3.4* (*At1G05200.1*), *GLR3.5* (*At2G32390*), and *GLR3.6* (*At3G51480*) relative to the reference gene *Tubulin 4* (*At5G44340*). Additionally, we confirmed the reduced expression of *GLR3.7* (*At2G32400.1*) in the mutant alleles using the same normalization method.

The results revealed a similar expression level for GLR clade 3 isoforms in both mutant alleles, *glr3.7-1* and *glr3.7-2*. *GLR3.7* exhibited a significant downregulation in both mutant backgrounds, the expression levels of other isoforms remained unchanged compared to WT (Fig 17). Despite this, a slight decrease expression was observed for *GLR3.4*, while *GLR3.1* showed a slight upregulation, although both trends were not statistically significant.

These findings indicate that the expression of no other GLR clade 3 isoform is modulated by GLR3.7. Surely, a Real-Time qPCR does not exclude that the lack of GLR3.7 might still impact the activity of other GLRs: In fact, it might lead to the formation of alternative heterotetrameric channels, resulting in a modified protein activity, localization or even degradation, thus, causing the observed phenotypes.

6.6. Rehydration of drought-stressed plants induce GLR3.3-dependent Ca^{2+} waves

As already mentioned, wounding and burning stresses applied to a single rosette leaf, trigger long-distance Ca^{2+} waves that propagate to distal tissues rapidly, in a mechanism dependent mainly on GLR3.3 activity (Bellandi et al., 2023; Farmer et al., 2014; Grenzi et al., 2023). Importantly, we showed that a similar long-distance Ca^{2+} wave was also induced when roots of plants grown in hydroponic culture were osmotically stressed, and specifically when a hypo-osmotic stress was applied (Grenzi et al., 2023). Importantly, when the hypo-osmotic stress was applied to roots of *glr3.3* mutant the long-distance Ca^{2+} wave in the rosette leaves was abolished (Grenzi et al., 2023).

Based, on the previous results obtained by studying the local AA administration and burning response in the *glr3.7* backgrounds we, thus, decided to test the effects of osmotic stress also on *glr3.7* mutants. However, we reasoned about the real physiological conditions in which a hypo-osmotic stress could be perceived by land plants. Such a condition may occur when drought-stressed plants are subjected to re-watering and, therefore, re-hydration. In order, to show that this is the case, and that plant re-

watering can induce long-distance Ca^{2+} wave we decided to develop a device, that could allow us to monitor the root apparatus during the development, monitoring the soil humidity as well as perform fluorescence imaging. Indeed, in literature similar systems have been reported (i.e. rhizotron) (Mathieu et al., 2015), but often they result cumbersome not fitting under a stereomicroscope, with limitations on growing media characteristics, mainly relying on hydroponics, and often limiting fluorescence-based imaging.

After multiple iterations and thanks to 3D design and 3D printing, we were able to design and make what we called mini-rhizotron who satisfied our requests, thus, allowing *in vivo* imaging of both aerial and root tissues of Arabidopsis plants during drought stress and re-hydration (Fig. 18.a). In our home-made device plants are grown in standard soil and, by exerting natural gravitropism, roots develop closely to a translucent sheet of acrylate polymer.

To study how drought-stressed plants respond to watering we grew Arabidopsis plants expressing the R-GECO1 in the mini-rhizotron for three weeks and then we withheld water until the soil got dry. At this stage we moved the plants under the stereomicroscope and performed *in vivo* imaging during re-watering. We recorded $[\text{Ca}^{2+}]_{\text{cyt}}$ dynamics, in four different genetic backgrounds, WT, *glr3.3-1*, *glr3.7-1* and *glr3.3-1/glr3.7-1* all expressing the R-GECO1 sensor. It should be noted that the two experiments were carried out in different instances, the first one was carried out to compare WT vs *glr3.3-1* and were performed by Matteo Grenzi, the second one was carried out by me to compare WT with *glr3.7-1* and *glr3.3-1/glr3.7-1* mutants. By watering WT plants with tap water (Fig 18.b and 18.d) we observed a robust rise in $[\text{Ca}^{2+}]_{\text{cyt}}$, spreading from the plant's base throughout the main vasculature of its whole aerial portion. Interestingly, this result agrees with our previous observations obtained with hydroponically grown osmotic-stressed plants (Grenzi et al., 2023), suggesting that such a signal propagation could be caused by a change in turgor pressure occurring in the main vascular bundles after water absorption from the soil.

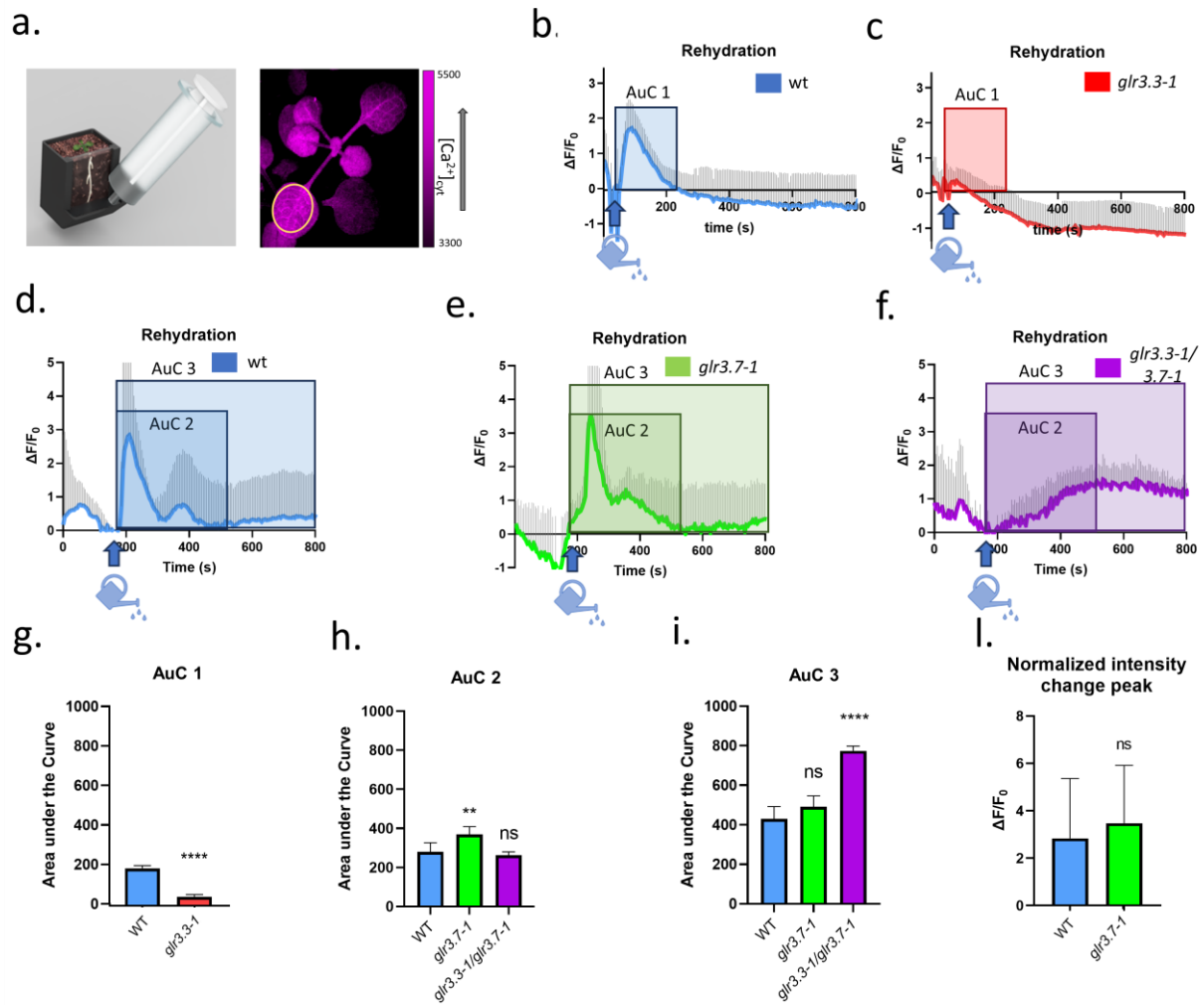


Figure 18 – Rehydration of GLR3.3 and GLR3.7 mutants. (a.) experimental setup for the rehydration protocol with a representative acquisition. In yellow the region of interest considered for the indicator analysis. (b.-c.) Representative curves of the R-GECO1 normalized intensity changes ($\Delta F/F_0$) acquired in response to the rehydration of the root apparatus applied at 60 s from the beginning of the measurement for the WT (b.) and *glr3.3-1* (c.) background. (d.-e.-f.) Representative curves from an independent experiment of the R-GECO1 normalized intensity changes ($\Delta F/F_0$) acquired in response to the rehydration of the root apparatus applied at 180 s from the beginning of the measurement for the WT (d.), *glr3.7-1* (e.) and *glr3.3-1/glr3.7-1* (f.) background. (d.) Area under the Curve (AuC) involving the *glr3.3-1* mutant rehydration. (h.-i.) Area under the Curve (AuC) involving the *glr3.7-1* and *glr3.3-1/glr3.7-1* mutant rehydration. Two intervals were selected based on the length of the response in *glr3.7-1* (h.) and *glr3.3-1/glr3.7-1* (i.) backgrounds. (j.) Normalized intensity increase peak of WT and *glr3.7-1* backgrounds. Error bars = SD. ns= not significant; ** $P < 0.01$, **** $P < 0.0001$ (Students *t* test in (g.) and One-way ANOVA test in (h.-i.)).

Whereas the *glr3.3-1* did not show any response, the *glr3.7-1* and the *glr3.3-1/glr3.7-1* mutants showed a response, however with different Ca^{2+} signature compared to the WT (Fig 18.d, 18.e, 18.f). Given the peculiar Ca^{2+} signatures observed with the different genetic backgrounds we decided to compare them by evaluation the area under the curve of the R-GECO1 indicator. This analysis revealed that while considering a time window compatible with WT and *glr3.7-1* Ca^{2+} signature (Fig 18.h) the double mutant did not exhibit statistically significant changes. However, considering a wider

range, consistent with *glr3.3-1/blr3.7-1* prolonged signature, revealed a significantly more sustained Ca^{2+} increase in the double mutant, compared to the WT and *blr3.7-1* backgrounds. More so, by performing a visual comparison of Ca^{2+} wave propagation we clearly observed other differences between WT and mutant responses. While the WT and *blr3.7-1* transmit the Ca^{2+} wave through the main vascular system (Fig 19.a and 19.c), extending to the secondary vascular system and leaf lamina, both *glr3.3-1* and *glr3.3-1/blr3.7-1* double mutant exhibits impaired signal propagation: Ca^{2+} elevations do occur, but fail to reach distal leaves vasculature. Instead, the highest elevation of cytosolic Ca^{2+} is detected at the level of the Shoot Apical Meristem (Fig 19.d).

The differences observed in the experiments performed by Grenzi (Fig 18.b, 18.c) and me are probably due to the intensimetric nature of the R-GECCO1 indicator, since its expression level and the nature of the tissue analysed heavily impact on the basal emitted fluorescence intensity. Plants from the second experiment, involving *blr3.7-1* and *glr3.3-1/blr3.7-1* mutant backgrounds were slightly older than those used in the first treatment, concerning the *glr3.3-1* mutant. The difference in the size, cell density and expression efficiency of the sensor could possibly explain the difference between the maximum normalized intensity changes displayed in the two independent WT lines (Fig 18.b and 18.e). Still, the shape of the curve of the WT backgrounds do not exhibit fundamental differences, proving the replicability of this procedure. Surely, the experiments need to be repeated with plants of all different genetic backgrounds grown in the same conditions.

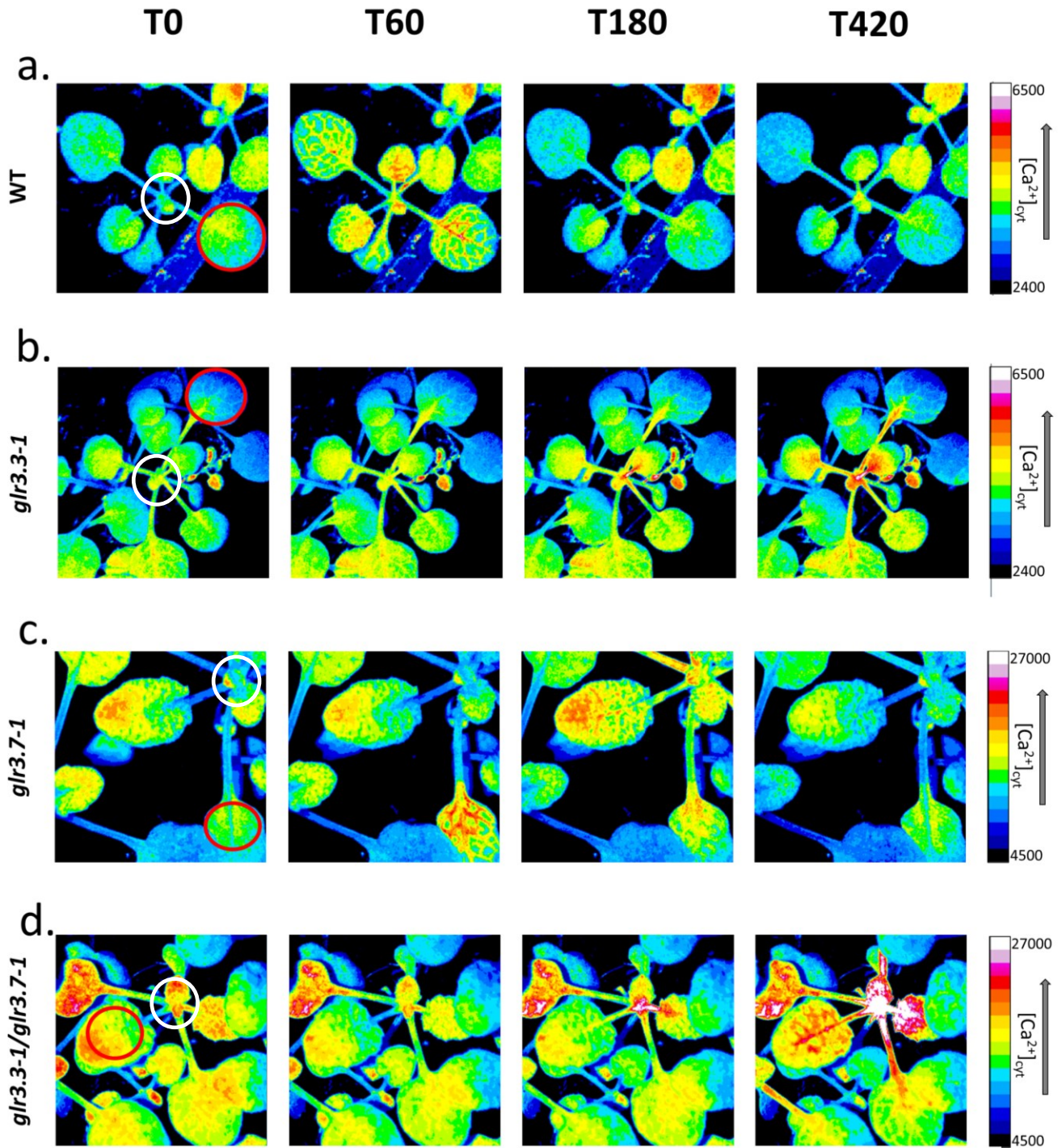


Figure 19 – Representative acquisitions of R-GECO1 fluorescence emission during rehydration in 16-colors LUT. (a.) WT response to rehydration (b.) *glr3.3-1* response to rehydration. (c.) *glr3.7-1* response to rehydration. (d.) *glr3.3-1/glr3.7-1* response to rehydration

Timepoints reported at 0, 60, 120 and 240 seconds after the application of the treatment. Highlighted in red the Region of Interest used to record representative curves of the R-GECO1 normalized intensity changes ($\Delta F/F_0$) acquired in response to the rehydration in figure 18. Highlighted in white the region containing the Shoot Apical Meristem (SAM).

In conclusion, we show that WT plants, after a period of drought stress, are able to perceive the presence of water and generate a long-distance Ca^{2+} signal in the shoot. Our interpretation of this result is that this process is linked to changes in vascular turgor pressure, which is a prerequisite for

the GLRs activation as previously suggested (Grenzi et al., 2023; Bellandi et al., 2023). Whereas GLR3.3 is required for the signal transmission, the GLR3.7 fine tunes its activity. Nonetheless, the consequences of such watering-triggered response need to be further investigated as well as the reason why there is the need to attenuate GLR3.3 activity.

7. Concluding remarks and future perspectives

Plants are remarkable examples of adaptability and resilience. Among the first organisms to colonize new environments, they rapidly adapt and develop in the most diverse conditions. Plants are constantly exposed to biotic and abiotic stress, an evolutionary pressure that has honed their ability to recognize and respond to these challenges through a sophisticated interplay of second messengers and phytohormones. When faced with an external stress, the local perception of this stress triggers long-distance signals propagation and distal responses in the whole organism, preparing the plant for further reiteration of the same damaging condition.

We recently showed that in *Arabidopsis* the mechanical damage of a rosette leaf induces a systemic accumulation of $[L-Glu]_{apo}$ and a subsequent $[Ca^{2+}]_{cyt}$ elevation, with the latter strictly mediated by GLR3.3 activity (Grenzi et al., 2023). Nevertheless, while GLR3.3 activity is a prerequisite for long distance propagation of $[Ca^{2+}]_{cyt}$ elevation, it is not necessary for $[L-Glu]_{apo}$ accumulation. Of interest, we showed that the local treatment with physiological concentrations of L-Glu was not sufficient in triggering a long-distance propagation of Ca^{2+} waves (Grenzi et al., 2023). This indicates that GLR3.3 is required for the of long-distance Ca^{2+} signals and initiating subsequent responses (Nguyen et al., 2018; Toyota et al., 2018; Bellandi et al., 2022; Grenzi et al., 2023) but is not sufficient to generate the propagation of the signal itself (Grenzi et al., 2023). In such a scenario we suggested that vascular turgor pressure changes, a mechanism compatible with the bulk flow model proposed by Bellandi et al. (2023), is possibly the driving force behind long-distance stress propagation. This model is supported by the demonstration that applying a local hypo-osmotic stress to roots, can generate responses similar to mechanical damage, inducing $[L-Glu]_{apo}$ accumulation and the GLR3.3-dependent $[Ca^{2+}]_{cyt}$ elevation. Taking all these pieces of information together, it is clear that GLRs, and particularly GLR3.3, function as potent molecular transducers of long-distance stress propagation. As such, a strict and delicate regulatory mechanism is necessary. GLR3.3 is an intriguing protein with unique characteristics: not only we demonstrated a higher ligand binding domain promiscuity compared to iGluRs (Alfieri et al., 2020), but that the ligand-binding is essential for the channel activation (Grenzi et al., 2023). As iGluRs, GLRs can form a functional pore through tetramerization of four subunits (Green et al., 2021) and some isoforms have been predicted to be able to hetero-tetramerize, modulating the pore selectivity and activity (Vincill et al., 2013; Price et al., 2013; Kong et al., 2016). This added layer of molecular plasticity is a critical factor in iGluRs activity and so could be a potent regulatory mechanism also in GLRs.

Among the other GLR isoforms, we have identified GLR3.7 as a putative regulator of GLR3.3. Specifically, GLR3.7 appears to act as a negative regulator of the GLR3.3 activity. By exploring previously published molecular responses dependent on GLR3.3 activity, we demonstrated that lack of GLR3.7 leads to a higher cytosolic Ca^{2+} elevation in response to exogenous AAs administration in seedling and in response to leaf burning in adult plants. The molecular influence of GLR3.7 seems to be epistatic on GLR3.3 activity as absence of GLR3.3 masks entirely GLR3.7 ablation. These results suggest a strict functional interaction between the two proteins. To test this hypothesis, we investigated a putative molecular interaction between the two. First, we considered the tissue-specific localization of the two proteins at the level of multiple stages and tissues. In seedlings, GLR3.3 and GLR3.7 are colocalized at the root tip, the site of AA perception, while in adult plants we can identify the two proteins be present at the level of floral tissues and phloematic vascular bundles. Delving deeper into the localization of the two proteins, a FRET-FLIM analysis in the heterologous system *Nicotiana benthamiana* has demonstrated a subcellular colocalization and close-proximity of GLR3.3-GFP and GLR3.7-mCherry, indicating a possible existence of a direct interaction of the two tagged proteins. In support of this hypothesis, we also showed that when expressed in yeast cells, GLR3.3 and GLR3.7 Co-Immunoprecipitate. Overall, this data strongly suggests that, at least in our experimental conditions, the two channels have the potential to interact. More so, whereas yeast cells expressing the GLR3.3 alone show growth defects, the expression of GLR3.7, together with GLR3.3, reverts this phenotype, suggesting that also in this heterologous system, the GLR3.7 exerts a negative role towards GLR3.3 activity. Finally, to correlate GLR3.3 and GLR3.7 activity in a physiological condition, we developed a protocol to perform Ca^{2+} imaging in adult Arabidopsis plants in response to re-hydration. In this condition, GLR3.3 was shown to be crucial for the propagation of long-distance Ca^{2+} signals, as its absence dramatically reduced the cytosolic Ca^{2+} increase observed in the rosette leaves upon watering. On the other hand, lack of GLR3.7 caused an exacerbation of GLR3.3-dependent responses, supporting again its negative regulatory role onto GLR3.3-dependent pathways.

Taking all these pieces of evidence together, we propose a model in which GLR3.3, as a keystone in Arabidopsis stress signalling propagation, feature a complex array of regulatory mechanism. GLR3.3 activity is mainly governed by its ligand-dependent gating, but to finely tune Ca^{2+} -mediated responses, a deeper layer of regulatory plasticity is probably necessary. In such a context one of the way the GLR3.3 activity is modulated depends on the ability to form hetero-tetrameric channels together with other GLR isoforms. Previous research showed that, in Arabidopsis ovules, GLR3.3 activity can be potentially modulated by GLR3.6 (Mou et al., 2020), but in this work we provide evidence in support a negative role of GLR3.7 in both local and systemic responses. Following these results, I would also like to deepen the characterization of GLR3.7 at the molecular level. To this end

I plan to assess the local and distal expression of different stress-responsive genes in two independent alleles for the GLR3.3 and GLR3.7, in response to a classical severe stress stimulus, like the already documented rosette leaf burning (Grenzi et al., 2023). Long-distance stress responses are not solely orchestrated by Ca^{2+} . During the next months I will also focus into the dissection of other second messengers influence on stress perception and responses. To this end I will employ the full set of lines that I have generated and selected during these 3 years of PhD (see Appendix 1). In particular, I generated a series of mutants (e.g. for the different GLR isoforms) (see Appendix 1) expressing different genetically encoded biosensors to monitor at the same time different parameters, such as Ca^{2+} and pH, Ca^{2+} and H_2O_2 , Ca^{2+} and redox potential of the glutathione pool. These lines, together with the new custom-built apparatus that I have designed will allow me to study the role of GLRs in long-distance stress signaling of different origin.

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Appendix 1 - Generation of Arabidopsis mutant lines expressing genetically encoded fluorescent indicator

Along the course of my PhD, the collaborations with various laboratories on different projects has taken me to explore a variety of second messengers involved in stress perception and response like Ca^{2+} , ROS, but also pH. Each of these parameters is equally important to the definition of tailored stress-mediated plant responses and so I also aimed to generate a portfolio of transgenic plants expressing multiple fluorescent indicators in different genetic backgrounds. Here I will report some of the lines I have produced:

Appendix table 1 – Transgenic lines expressing different fluorescent indicators for Ca^{2+} (R-GECO1, mCherry-GCaMP6f), H_2O_2 (HyPer7, roGFP2-Orp1), GSH/GSSG pool (Grx1-roGFP2) and pH (E2GFP). In green the lines selected and tested, in yellow the lines currently in selection. R-GECO1 (Zhao et al., 2011), mCherry-GCaMP6f (Weigand et al., 2021), HyPer7 (Pak et al., 2020), roGFP2-Orp1 (Nietzel et al., 2019), Grx1-roGFP2 (Meyer et al., 2007), E2GFP (Bizzarri et al., 2006).

	Line ID	R-GECO1	mCherry-GCaMP6f	HyPer7	roGFP2-Orp1	Grx1-roGFP2	E2GFP
<i>WT</i>	Col0	v	v	v	v	v	v
<i>glr3.3-1</i>	Salk_040458	v	v	v	v	v	v
<i>glr3.6-a</i>	Salk_091801	v		v	v		
<i>glr3.7-1</i>	Salk_022757	v	v	v	v	v	v
<i>glr3.3-1</i> <i>/glr3.7-1</i>	Salk_040458 <i>/Salk_022757</i>	v					v
<i>rbohD</i>	Salk_JP65_4B 03L	v		v	v		
<i>rbohF</i>	Nasc_9557	x		v			
<i>hpcal-1</i>	CS923304	v		v	v		
<i>nadkc</i>	Gabi_3hh11			v			
<i>nadkc</i>	Nasc_006202			v			
<i>gr1-1</i>	Salk_105794	v		v			

9. List of scientific publications on Journals with Impact Factor

Long-distance turgor pressure changes induce local activation of plant glutamate receptor-like channels.

Grenzi M., **Buratti S.**, Parmagnani A.S., Aziz I.A., Wojcik I.B., Resentini F., Šimura J., Doccu F.G., Alfieri A., Luoni L., Ljung K., Bonza M.C., Stavriniidou E. Costa A. 2023.

Current Biology, 2023 | 33: 1019-1035 DOI: 10.1016/j.cub.2023.01.042

Author contributions:

Alex Costa and Matteo Grenzi conceived the project. Alex Costa and Matteo Grenzi designed the experiments. Matteo Grenzi, Ambra Selene Parmagnani, Ilaria Abdel Aziz, Francesca Resentini, Fabrizio Gandolfo Doccu and I performed and analysed the experiments. Ambra Selene Parmagnani generated the phloem localized Cameleon line. Ilaria Abdel Aziz, Iwona Bernacka-Wojcik and Eleni Stavriniidou developed the OEIP device. Jan Šimura and Karin Ljung carried out the mass spectrometry measurements and analysis. Laura Luoni generated the complemented transgenic lines. Matteo Grenzi prepared the figures and videos. Andrea Alfieri designed and illustrated the GLR3.3 LBD mutation strategies and prepared [Figure S1](#). Alex Costa. and Maria Cristina Bonza wrote the manuscript with input from all authors. I performed and analysed part of the experiments displayed in the manuscript.

First, I quantified the distal expression of stress marker genes *JAZ10* and *ZAT12* and the “stem bending” in WT, *glr3.3-1* and *glr3.3-2* mutant backgrounds after a local burning stress. I designed and 3D printed the imaging platforms for real-time hypo-osmotic stress administration in hydroponically grown adult Arabidopsis plants.

I also quantified the expression of *GLR3.3* in the WT, mutant and complemented backgrounds and analysed the tissue specificity of *GLR3.6* expression in Arabidopsis inflorescence through the transcriptional reporter *pGLR3.6::GUS*.

Plant Ca^{2+} -ATPases: From biochemistry to signalling.

Costa A., Resentini F., **Buratti S.**, Bonza M.C. 2023.

Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2023| 1870: 119508 DOI:
10.1016/j.bbamcr.2023.119508

Author contribution:

Maria Cristina Bonza and Alex Costa conceived the project and wrote the article with feedback from all authors. Francesca Resentini wrote specific portions of the text. I prepared all the figures featured in the article and participated in the revision of the manuscript.

Mitochondria-derived reactive oxygen species are the likely primary trigger of mitochondrial retrograde signaling in Arabidopsis.

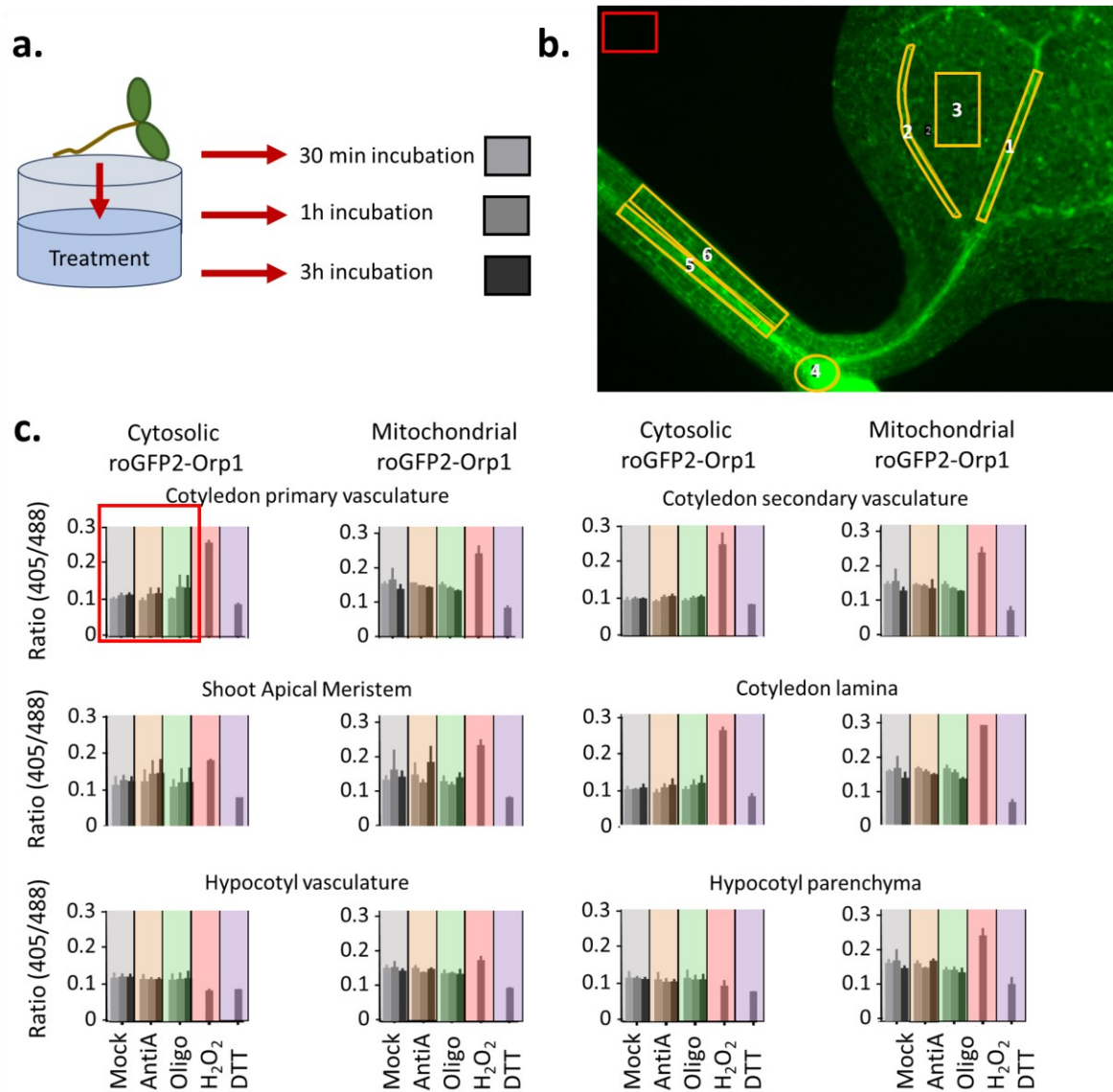
Khan K., Tran H.C., Mansuroglu B., Önsell P., **Buratti S.**, Schwarzländer M., Costa A., Rasmusson A.G., Van Aken O. 2024.

Current Biology, 2024 | 34: 1-18, DOI: 10.1016/j.cub.2023.12.005

Author contributions

Olivier Van Aken, Kasim Khan, Allan G. Rasmusson, Markus Schwarzländer, and Alex Costa conceived and planned the project. Kasim Khan, Huy Cuong Tran, Berivan Mansuroglu, Pinar Önsell, and performed the presented experiments. Olivier Van Aken and Kasim Khan analysed data. Olivier Van Aken and Kasim Khan wrote the manuscript with input from all co-authors. I performed experiments in Arabidopsis seedlings to define the tissutal origin of the ROS accumulation characterized in the plate reader analysis. My experiments were not selected for the final cut of the manuscript, but here I report some of the results we obtained.

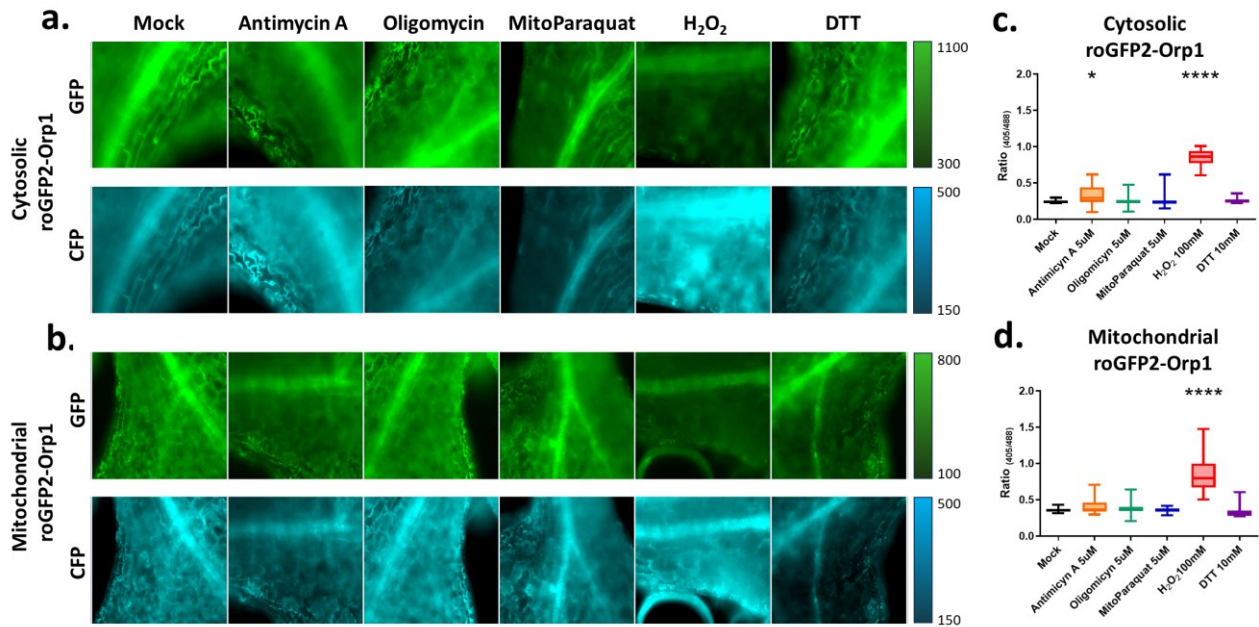
Our main aim was to localize the tissutal origin of the reported ROS increase after treatments known to impact on the plant Redox state, like Antimycin A, Oligomycin, H₂O₂ and DTT. To this end we analysed whole seedlings after 30 minutes, 1 hour and 3 hour of incubation in an imaging solution (composition reported in Materials and methods) supplied with 5 µM antimycin A, 5 µM Oligomycin, 100 mM H₂O₂ or 10 mM DTT (Supplementary figure 1.a). We then focused on 6 regions of interest, the cotyledon primary and secondary vasculature, the lamina, the region containing the Shoot Apical Meristem and the hypocotyl vascular bundle and parenchyma. To do so, we had to limit the acquisition at low magnification, 4X, increasing the field of view, but losing some definition (Supplementary figure 1.b). Here we report a strong response, in terms of reported H₂O₂ concentration ([H₂O₂]) change, to the exogenous H₂O₂ in most tissues analysed, but we did not detect any statistically relevant change in the other treatments (Supplementary figure 1.c). Notably, we detected an interesting trend in the reported oxidation of the cytosolic roGFP2-Orp1 indicator at the level of the cotyledon primary vasculature, both when treating the sample with Antimycin A and Oligomycin and increasing with time.



Supplementary figure 12 – Whole seedling treatment and cytosolic/mitochondria ROS monitoring. (a.) experimental setup of the incubation protocol. (b.) Indicative acquisition at 4X magnification reporting regions of interest (ROI) measured: 1, Cotyledon primary vasculature; 2, Cotyledon secondary vasculature; 3, Cotyledon lamina; 4, Shoot apical meristem region; 5, Hypocotyl vascular bundle; 6, Hypocotyl parenchyma; in red also reported the background. (c.) Mean ratiometric response of cytosolic roGFP2-Orp1 reporter and mitochondrial roGFP2-Orp1 reporter in the analysed ROIs. In red reported the only consistent trend observed (not statistically significant).

Following this result, we repeated the analysis focusing on the cotyledon primary vasculature of treated seedlings. To exacerbate the response we incubated the seedlings for 6 hours and to obtain a better reading of the indicator response we increased the magnification to 20X. With this setup, we detected a clear difference between the two reporters: the mitochondrial roGFP2-Orp1 did only report a mitochondrial [H₂O₂] increase in response to exogenous H₂O₂, but not to the other treatments, while cytosolic roGFP2-Orp1 did also report an increase in cytosolic [H₂O₂] levels after Antimycin A treatment (Supplementary figure 2.c). While this result does support the results presented in the

manuscript, obtained with a plate reader, it did not report any activity of Oligomycin or Mito-Paraquat on mitochondrial and cytosolic ROS homeostasis, possibly suggesting that our approach was less sensitive compared to the Plat Reader assay.



Supplementary figure 13 – Seedling primary vasculature cytosolic/mitochondria ROS monitoring. (a.) Split channel indicative acquisitions at 20X magnification of cytosolic roGFP2-Orp1 response to the different treatments applied. (b.) Split channel indicative acquisitions of mitochondrial roGFP2-Orp1 response to the different treatments applied. (c.) mean ratiometric response of cytosolic roGFP2-Orp1 in response to 6 hours incubation in the different treatments at the level of the primary vasculature. (d.) mean ratiometric response of mitochondrial roGFP2-Orp1 in response to 6 hours incubation in the different treatments at the level of the primary vasculature. Error bars = SD. * $P < 0.05$, **** $P < 0.0001$ (One-way ANOVA test).

AMEBaS: Automatic Midline Extraction and Background Subtraction of Ratiometric Fluorescence Time-Lapses of Polarized Single Cells.

Badain R., Damineli D. S. C., Portes M.T., Feijó J., **Buratti S.**, Tortora G., Neves de Oliveira H., Cesar R. M. 2023.

Journal of Visualized Experiments, 2023.| 23:(196) DOI: 10.3791/64857

Author contributions:

Rafael Badain, Daniel S. C. Damineli, Maria Teresa Portes, José Feijó, Hugo Neves de Oliveira and Roberto M. Cesar Jr. conceived the project and developed the presented pipeline. In the reported research, I, in collaboration with Giorgia Tortora, mainly provided the samples used to test and tailor the computational analysis pipeline.

10. Other Scientific publication (e.g. Book chapters, non-IF journals)

Non-invasive in planta live measurements of H₂O₂ and glutathione redox potential with fluorescent roGFPs-based sensors

Buratti S, Grenzi M, Tortora G, Nastasi SP, Dell’Aglia E, Bassi A, Costa A, 2024.

Methods Mol Biol. 2024;2798:45-64. DOI: 10.1007/978-1-0716-3826-2_4.

Author contribution:

Alex Costa conceived and planned the project. Alex Costa, Andrea Bassi, Matteo Grenzi, Elisa Dell’Aglia and I wrote the manuscript and reviewed the chapter. In this chapter, as first author, I performed the majority of the experiments reported, analysed the data, designed the figures and the tables featured in the manuscript. I was also supported by Matteo Grenzi, Giorgia Tortora and Sara Paola Nastasi in the acquisition and analysis of the data presented. I characterized the genetically encoded indicators response in our experimental setup to H₂O₂ and DL-Dithiothreitol (DTT) treatments. I also 3D printed the imaging platforms used during acquisition and reported the model case study of redox status in different growing conditions of hydroponically grown Arabidopsis plants. Andrea Bassi and Giorgia Tortora developed the analysis pipeline featured in the article.