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Role of Rho GTP-ase nanodomains in regulation of plant's cell signalization

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie, Intégrations, Diversité Adaptative des Plantes

École doctorale GAIA

Unité de recherche Biochimie et Physiologie Moléculaire des Plantes

Rôle des nanodomains de Rho GTPase dans la régulation de la signalisation cellulaire chez les plantes

Présentée par SMOKVARSKA Marija

Le 11 Décembre 2020

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Abstract

(English)

Continuous perception and rapid reaction to their environment are crucial features of the plants in the course of their growth and development. To maintain the water status and acclimate to environmental constraints, plants have developed short-term (fast regulation of stomatal aperture and tissue hydraulics) and long-term (alteration of root system architecture and leaf abscission) responses. Despite their central role, the early cellular events that lead to these adaptive responses are largely unknown. Whereas the molecular bases of plant osmotic perception are not fully characterized, reactive oxygen species (ROS) convey the first cellular response and are crucial secondary messengers during osmotic signaling. The main question of this Ph.D. thesis is to dissect the molecular transduction and, ultimately, the perception of osmotic stimuli. The cell plasma membrane is the major coordinator of many environmental signaling complexes. In the context of hyperosmotic constraints, the cell responds by accumulation of Reactive Oxygen Species (ROS), produced enzymatically by Respiratory Burst Oxidase Homolog (Rboh), and an additional pathway involving apoplastic ascorbate and iron (Martiniere et al., 2019). By combining a gene candidate approach and varieties of microscopy technics, we demonstrated that the Arabidopsis Rho GTPase Rho of Plants 6 (ROP6) is the upstream regulator hub of the two ROS producing agents and that it is necessary to some downstream plant responses to osmotic stress. Furthermore, we demonstrated the mechanism by which ROP6 can directly regulate ROS production. ROP6 can recruit its effectors, RbohD and RbohF, in “osmotic specific” nanodomains on the PM. Whereas it is known that ROP6 participates in auxin signaling and that this hormone induces ROP6 nanodomain formation, the Rboh effector proteins are absent of these nanodomains. Consequently, auxin does not induce ROS accumulation in cells. This shows that nano-organization of a single ROP isoform in the membrane can encode for signal specificity. This work also demonstrated a crucial role of one of the activators of the ROPs in the GTPase cycle, guanine nucleotide exchange factor 14 (GEF14). It appears that GEF14 could be the specific activator of ROP6 in osmotic stressed environments. Small GTPases are classically acting downstream of membrane receptors. In plants, *Catharanthus roseus* Receptor-like kinases (CrRLKs) are known to participate in the perception of the cell wall environment that is strongly impacted during hyperosmotic stress. By using live imaging methods, we demonstrated that Feronia, a typical CrRLK homolog, is necessary but not sufficient for osmotic signaling by acting indirectly on ROP6 signaling and regulating its diffusion. Further studies revealed that anionic lipid phosphatidylserine (PS), which has been described to fine-tune the spatiotemporal dynamics of small GTPases (Platre et al., 2019), can be regulated by Feronia. Our observation suggests that Feronia is able to act quantitatively on ROP signaling both in roots and shoots, through the regulation of PS amount and clustering at the PM, therefore serving as a rheostat for the small GTPase signaling.

In summary, this work sheds some light on part of the molecular machinery of the osmotic signaling cascade in plants, where the small GTPase and cell wall sensing machinery refine the regulation of protein localization and dynamics within the membrane.

(Français)

Chez les plantes, une perception continue de l'environnement est cruciale pour la croissance et le développement. Dans le cas de la contrainte hydrique, les plantes ont développé des réponses à court terme (régulation rapide de l'ouverture stomatique et de l'hydraulique tissulaire) et à long terme (altération de l'architecture du système racinaire et abscission foliaire). Malgré leur rôle central, les événements cellulaires précoces qui conduisent à ces réponses adaptatives sont largement inconnus. Les espèces réactives de l'oxygène (ROS) représentent une des premières réponses cellulaires et sont des messagers secondaires cruciaux lors de la signalisation osmotique. Ainsi, la principale question de cette thèse est de disséquer la transduction moléculaire en amont de la production de ROS et, *in fine*, la perception du stimulus osmotique.

La membrane plasmique (MP) est le principal coordinateur de nombreux complexes de la signalisation environnementale. La cellule répond à la contrainte hyperosmotique par accumulation d'espèces réactives de l'oxygène (ROS) produites par les enzymes de la famille des Rboh (Respiratory Burst Oxidase Homolog) et une voie additionnelle qui associe le fer apoplastique et sa réduction par l'ascorbate (Martiniere et al., 2019). En combinant des approches génétiques et des techniques de microscopie, nous avons démontré que la Rho GTPase ROP6 (Rho of Plants 6) d'*Arabidopsis* est nécessaire et suffisante pour la production de ROS dans des cellules sous stress osmotique. De plus, nous avons montré que ROP6 contrôle plusieurs réponses cellulaires et tissulaires en aval de la production de ROS. Nous avons ensuite démontré le mécanisme par lequel ROP6 peut réguler directement la production de ROS. ROP6 recrute ses effecteurs, RbohD et RbohF, dans des nanodomains spécialisés de la MP. On sait que ROP6 participe également à la signalisation de l'auxine. Alors que cette hormone induit la formation de nanodomains ROP6, les protéines effectrices Rboh sont absentes de ces nanodomains. Aussi, l'auxine n'induit pas d'accumulation de ROS dans les cellules. Cela montre que la nano-organisation dans la membrane d'une même isoforme de ROP détermine la spécificité du signal. Ce travail a également démontré le rôle crucial de l'un des activateurs des ROP dans le cycle GTPase, le facteur d'échange de nucléotides guanine 14 (GEF14). Il semble que GEF14 pourrait être l'activateur spécifique de ROP6 dans un environnement soumis à un stress osmotique. Les petites ROPs agissent classiquement en aval des récepteurs membranaires. Chez les plantes, on sait que les récepteurs kinases de la famille *Catharanthus roseus* (CrRLK) participent à la perception de l'environnement de la paroi cellulaire qui est fortement impacté lors d'un stress hyperosmotique. En utilisant des méthodes d'imagerie à super-résolution, nous avons démontré que Feronia, un membre typique de la famille CrRLK, est nécessaire mais pas suffisant pour la signalisation osmotique en régulant indirectement la diffusion de ROP6. D'autres études ont révélé que la phosphatidylsérine (PS), un lipidique anionique connu pour affiner la dynamique spatio-temporelle des ROPs (Platre et al., 2019), peut être régulée par Feronia. Nos observations suggèrent que Feronia est capable d'agir quantitativement sur la signalisation ROP grâce à la régulation de la quantité de PS à la membrane et son regroupement en nanodomains. Elle agit donc comme un rhéostat sur la signalisation ROPs. Ce travail a révélé l'importance de la dynamique des protéines au sein de la MP pour le fonctionnement de la signalisation Rho GTPase des plantes que ce soit pour déterminer la spécificité des signaux comme pour les intégrer.

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I've read somewhere that a PhD thesis "takes a village". That is the truest statement ever related to a PhD work; therefore, this is for all, "my village" people.

I have to apologize in advance for this part is long; it has tremendous meaning me so I'll take my time.

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I dedicate this thesis to her.

*“Chose a job you love, and you will never have to work a day in your life”-
Confucius*

I surely did.

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List of abbreviations:

ABA-Abscisic acid

ATHK1-ARABIDOPSIS HISTIDINE KINASE 1

BFA-Brefeldin A

BIK1-BOTRYTIS-INDUCED KINASE 1

C2LACT-C2 Lactadherine

CAMTA- CALMODULIN-BINDING TRANSCRIPION ACTIVATORS

CBL-CALCINEURIN-B LIKE PROTEINS

CESA-CELLULOSE SYNTHASE

CHO-Chinese hamster ovary

CIPKs-CBL-INTERACTING PROTEIN KINASES

CMLs-CALMODULIN-LIKE PROTEINS

CPKs-Ca²⁺-DEPENDENT PROTEIN KINASES

CW-cell wall

CWD-cell wall damage

CWI-cell wall integrity

CZH-CDM-zizimin homology

DEK1-DEFECTIVE KERNEL 1

DHE-Dihydroethidium

DMSO-Dimethylsulfoxide

DRM-Detergent resistant membrane

EM-electron microscopy

EMS-Ethyl methane sulphonate

ER-Endoplasmic reticulum

EVCT2 ERECTIN 2

EXT3-EXTENSIN 3

FAPP1 FOUR PHOSPHATE ADAPTOR PROTEIN 1

FER-FERONIA

Flg22- Flagelin 22

FLIM-Fluorescence lifetime imaging

FLS2-Flagelin sensitive 2

FM4-64-(Diethylamino)Phenyl)Hexatrienyl)pyridinium dibromide

FRET-Förster resonance energy transfer

GAP GTPase-activating proteins
GDI-Guanosine nucleotide dissociation inhibitor
GDP-Guanosine diphosphate
GEF Guanine nucleotide exchange factors
GIPC-Glycosyl Inositol Phospho Ceramides
GTP-Guanosine triphosphate
HOG-high osmolarity glycerol
HRV-Hyper variable region
ISX-isoxaben
K-Ras-V-KI-RAS2 KIRSTEN RAT SARCOMA VIRAL ONCOGENE HOMOLOG
LGG-LORELEI (LRE)-LIKE GLYCOSYLPHOSPHATIDYLINOSITOL (GPI)-ANCHORED
PROTEIN
LPA-Lyso phosphatidic acid
LPC-Lyso phosphatidylcholine
LPS-Lyso phosphatidylserine
LRX- LRR-EXTENSIN
MAPK-MITOGEN ACTIVATED PROTEIN KINASE
MCSL-mechanosensitive channels of small conductance-like
MCA-mid1-complementing activity
MSL-MscS-like
MS-mechanosensitive channels
MT-Microtubule
NAA-Naphthaleneacetic acid
NADPH-Nicotinamide adenine dinucleotide phosphate
ORP-OSBP-RELATED PROTEIN
OSBP-OXYSTEROL BINDING PROTEIN
OSCA1-REDUCED HYPEROSMOLARITY-INDUCED Ca²⁺ INCREASE 1
PA-Phosphatidic acid
PAT-PROTEIN S-ACYL TRANSFERASE
PBR-Polybasic region
PC-Phosphatidylcholine
PE-Phosphatidylethanolamine
PH-Pleckstrin homology
PI4P-Phosphatidylinositol-4-phosphate

PIN-AUXIN EFFLUX CARRIER
PIP2;1-PLASMA MEMBRANE INTRINSIC PROTEIN
PIP-Phosphatidylinositol phosphate
PLD-PHOSPHOLIPASE D
PM-Plasma membrane
PRC-PROCUSTE
PS-Phosphatidylserin
PVCs-Pavement cell
RALF-Rapid alkalization factor
RBOH-RESPIRATORY BURST OXIDASE HOMOLOG
RHD4-ROOT HAIR DEFECTIVE MUTANT 4
RIC-ROP INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN 4
RLK-RECPETOR-LIKE KINASE
ROP-RHO OF PLANT
ROS-Reactive oxygen species
SnRK-SNF1 RELATED PROTEIN KINASE
SOS1- SALT OVERLY SENSITIVE 1
SPK-SPIKE
SptPALM-Single particule tracking photo activated localization microscopy
TGN-*trans*-Golgi network
THE-THESEUS
TIRF-Total internal reflexion microscopy
TMD-trans membrane domain
TMK-TRANSMEMBRANE KINASE
TPK-Two-pore potassium
WAK-WAL ASSOCIATED KINASE

1. General introduction

1.1 Background

Despite being fixed in a singular geographic location determined by the site of germination, plants regulate their growth in ways that allow them to extend millimeters to meters out into their environment to survive and thrive. Such growth regulation is orchestrated by the environmental cues. From fluctuations in temperature, light, ions, metabolites, water availability to pathogen attacks, plants sense and adapt (Rout and Das, 2013). Eyes, ears and a nervous system in general are structures that we intuitively associate when thinking about environment perception. Plants lack these structures and yet they are able to “be aware” of their surroundings. One major axis in plant biology is to understand how plants can sense their environment and how they can in return regulates their physiology and development. Throughout the decades extensive studies have been made, in this regard, that shed a glimpse of light on plant perception.

1.2 Plant water stress

Water is one of the most limiting abiotic factors that have great impact on plants growth and development.

On a whole plant level, in response to water stress, plants will reduce their life cycle (Sakamoto et al., 2008), limits their evapotranspiration by leaf abscission (Sakamoto et al., 2008), change their root architecture to optimize water foraging (Deak and Malamy, 2005) and modify their roots structures by producing more impermeable components like lignin and suberin (Geng et al., 2013). Those responses are considered long term responses produced after several days. Other kind of responses could happen faster, such as adjustment in the cell shape and growth (Craddock et al., 2012). Changes in the cell wall properties lead to osmotic adjustment and this can help facilitate water uptake into cells to maintain growth under water deficit (Dinneny et al., 2015). Other fast responses that happen shortly after osmotic stress are stomata closure (Islam et al., 2016), the modification of root hydraulic (for example, the water conductivity (Boursiac et al., 2005) and last but not least, modification in the endocytosis mechanism, which is considered to be one of the fastest response to an osmotic stress. Only in few tens of minutes after applying an osmotic treatment (sodium chloride or mannitol), the balance between endocytosis and exocytosis is adjusted maybe to maintained plant membrane integrity during cell volume adjustment (Luu et al., 2012; Zwiewka et al., 2015).

The plethoras of these responses are downstream events where plants need to perceive and transduce the water signal.

1.2.1 Cellular water status and the water signal

The plant cell is composed of an aqueous cytoplasm surrounded by a plasma membrane and a cell wall. The membrane is permeable to the movement of small, uncharged molecules such as water, but is relatively impermeable to the movement of many dissolved solutes (Kramer and Boyer, 1995). As a result, an increase in solute concentration on one side of the membrane creates a water movement by osmosis from the less concentrate to the more concentrate compartment until the dissipation of the osmotic gradient. The tendency of water to flow into a particular compartment is quantified by water potential (Kramer and Boyer, 1995), which is the potential energy of water per unit area compared to pure water. The water potential gradient between two compartments acts as the driver for water movement: water flows from areas of high potential to low potential. Water will continue to move between the cell and its outside space until the two come to water potential equilibrium. Swelling of the cell owing to water flow is largely prevented by the presence of a rigid cell wall, which resists deformation and allows buildup of intracellular hydrostatic pressure (turgor) of several atmospheres which largely supports the upright shape of the plant (Kramer and Boyer, 1995). Thus, the total water potential of a cell is the sum of both osmotic potential, dictated by the concentration of solutes within the cell, and pressure potential, caused by pressure against the cell wall.

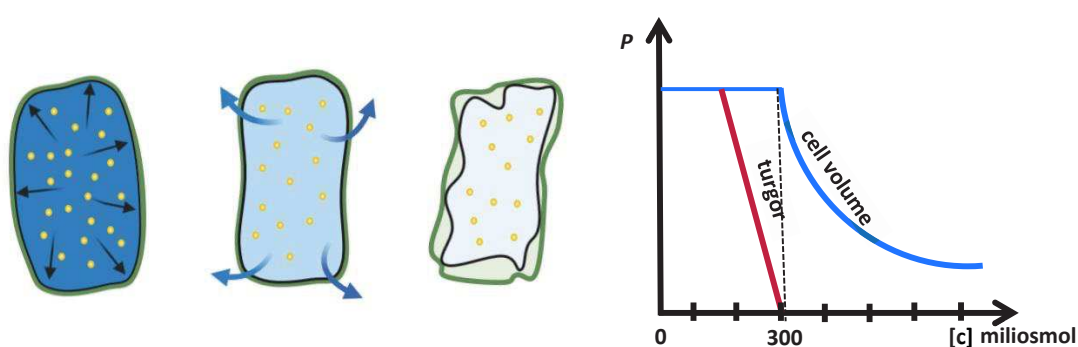


Figure1. **Water potential is the main driving force for water movement in cells.**

- (A) In non-stressed cells, is under full turgor pressure (black arrows) that is due to the intracellular solute concentration (yellow circles) which is below the external water potential. (left). During water stress, when water start leaving the cell due to increased extracellular solute

concentration, the turgor pressure decreases which leads to changes in cell volume (shrinkage) (center) and in severe cases, detachment of the membrane from the cell wall (incipient plasmolysis) (right).

- (B) Graphical representation of the relationship between the pressure and solute concentration. At low osmoconcentrations both the turgor and the cell volume can remain relatively stable. But at a certain concentration (300miliosmol) the turgor drops rapidly and when decreases completely, then the cell starts to lose its volume (as indication 300mOsmol confers incipient plasmolysis to root epidermal cell in elongation).

The water signal induced by water shortage is for a part osmotic. Indeed, the decrease of the soil water potential will reduce the osmotic gradient between cell exterior and interior, inducing first a decrease of cell turgor. Then, as soon the turgor is null, cell will experience a drastic change of their volume that is called plasmolysis caused by an efflux of water.

But, the osmotic stimulus is not the only component of the water signal. For instance, the mechanic propriety of soil changes depending of its moisture. This mechanical change in the vicinity of the root could also be perceived by the plant cells. In addition, during drought period, plant perceives a shortage of water but not only. Scarcity of nutrients is a side effect of the water deficit, since the water might conduct ions. Therefore, it is obvious that different sensing mechanisms are activated in plants during water stress. To simplify this multifactorial stress, we have decided to focus, the topic of my PhD, to one of the signals induce by water shortage, the osmotic signal.

1.3 Osmotic perception

The changes in osmotic balance have important effect on the plant physiology. These changes happen during many abiotic stresses such as cold or water stress but also during normal development like for cell expansion or seed germination. But despite the examples of how osmotic imbalance triggers vast responses, the sensing mechanisms of the osmotic stimuli are still largely unknown. Based on the supporting experimental evidence there are several models for osmosensing in plants.

1.3.1 Osmotic imbalance between the two sides of PM

If we imagine the structural properties of transmembrane protein, one would say that these embedded PM proteins can directly sense the changes of the osmolarity inside and outside of the cell. Initial knowledge that comes from osmosensing mechanisms in yeast may favor this hypothesis. To cope with external high osmolarity, the budding yeast activates the Hog1 MAPK through the high osmolarity glycerol (HOG) signaling pathway (Hohmann et al., 2002).

As the name states, the probably most important role of the HOG pathway in osmotic adaptation concerns the control of glycerol accumulation. Glycerol serves as the osmolyte of proliferating yeast cells (Hohmann et al., 2002). It performs this role by partly replacing water and protecting biomolecules inside the cell as well as by increasing the intracellular water potential and thereby driving back water into the cell. Upstream of the HOG pathway one sub branch goes to the osmosensor suppressor of *nup116-C* lethal SLN1 histidine kinase (Posas et al., 1996). SHO1 is another upstream regulative cascade of the HOG pathway. It has long been believed to be the sensor in the system (Maeda et al., 1995). However, it appears to play a role as membrane-localized scaffold protein that recruits components to the cell surface at places of active cell surface growth and remodeling (Reiser et al., 2000). A cDNA for a hybrid-type histidine kinase that has structural similarity to the yeast osmosensor SLN1 pointed to ATHK1 as putative plant osmosensor. The ATHK1 protein contains both a transmitter and a receiver domain with conserved histidine and aspartate residues, respectively, and a transmembrane domain with two hydrophobic regions toward the N terminus, as does SLN1 (Urao et al., 1999). Overexpression of Arabidopsis histidine kinase AHK1/ATHK1 in yeast *sln1* and *sho1* deletion mutants enables the yeast mutant to grow normally under high-salinity conditions, suggesting that the histidine kinase AHK1 can sense and transduce a signal of external osmolarity to downstream targets (Tran et al., 2007). However, because loss of function of this kinase results in mild effect on key phenotypes such as accumulation of ABA or osmoregulatory solutes, other osmosensory systems might exist (Kumar et al., 2013).

1.3.2 Mechanical tension of the PM

Due to high turgor pressure within the plant cell, the PM is pushed against the cell wall. As result, both cell wall and plasma membrane are under tension. During osmotic shock (either hypo or hyper), this tension vary, before any deformation of cell protoplast. Mechanosensitive channels (MS) alter their conductivity to ions in response to changes in membrane tension and therefore can convert mechanical signals to ion fluxes. Multiple families of MS ion channels have been identified in plant genomes, and numerous MS ion channel activities have been detected in plant membranes. There are 5 main families of MS ion channels have been identified: mid1-complementing activity (MCA), mechanosensitive channels of small conductance-like (MSL), Reduced Hyperosmolality-Induced $[Ca^{2+}]$ increase1/Calcium permeable stress gated cation channel (OSCA1/CSC1), PIEZO and two-pore potassium (TPK) families (Monshausen and Haswell, 2013, Hamant and Haswell, 2017) (Figure 2).

The Ca^{2+} MS channels like MCAs are involved in sensing both hyper and hyposomotic shock.

MCA1 was isolated from a genetic screen of Arabidopsis cDNA, which complements the lethality of yeast mutant lacking MID1 gene, which is a MS channel (Nakagawa et al., 2007). MCA1 promotes Ca^{2+} influx leading to ROS generation in response to hyposmotic shock and mechanical stimulus in rice and tobacco, as well. (Kurusu et al., 2012).

One of the best characterized Ca^{2+} channels that have role in the osmotic signaling is mechanosensitive OSCA1. OSCA1 is Ca^{2+} permeable channel identified from an ethyl methane sulphonate (EMS) screen searching for defects in hyperosmolality-induced Ca^{2+} increase (OICI) and described by electrophysiological studies in Chinese Hamster Ovary

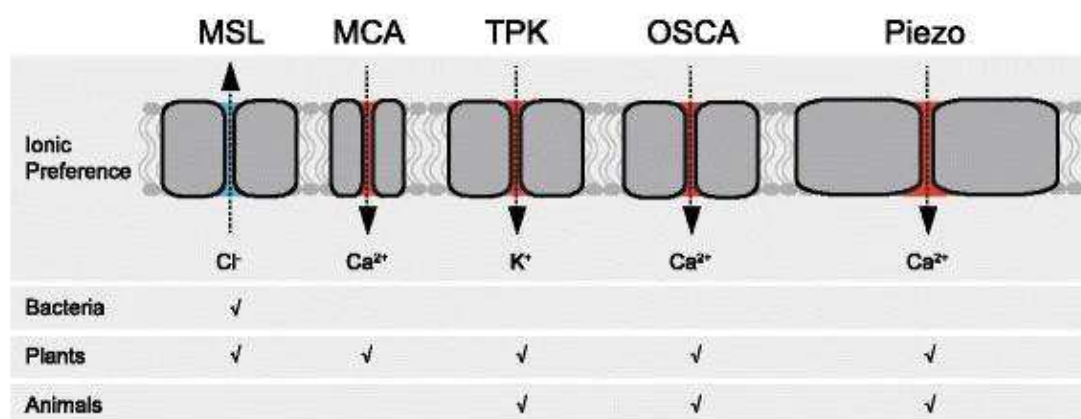


Figure 2: **Families of likely plant mechanosensitive ion channels.** From left to right: MscS-like (MSL), Mid1-Complementing Activity (MCA), Two Pore Potassium (TPK), Reduced hyperosmolality-induced $[\text{Ca}^{2+}]$ increase (OSCA), and Piezo channel families, with their proposed primary ion permeability. The presence of homologs in bacterial, plant, and/or animal genomes is indicated with a checkmark. The predominant ion flux is shown for each channel. Adapted from Hammant and Haswell, 2017

(CHO) cells (Yuan et al., 2014, Chou et al., 2014). *osca1* mutants, displays impaired osmotic Ca signaling in guard and root cells, and attenuated water transpiration regulation and root growth in response to osmotic stress (Figure 3). (Yuan et al., 2014). Murthy et al., 2018 characterized that OSCA1 are bona-fide, pore-forming mechanosensitive channels because transfection of various members of the family from plants, flies, and mammals give rise to robust mechanically activated currents. The Cryo-EM structure of OSCA1.2 revealed two hook-shaped loop that enter the membrane and slightly deforms the lower leaflet. Their association with the cytoplasmic bilayer leaflet suggests these two features could be sensitive to membrane tension, and their movement could be coupled to channel conformation through their interactions with the TMD. Membrane tension promotes gating by affecting the lipid occupancy on the cytoplasmic side and lipids occupy and occlude the cytoplasmic half of OSCA1. Their MD simulations of OSCA1.2 in a phospholipid (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, POPC) bilayer, shows lipid molecules consistently placed inside the gap, indicating a role for lipids in stabilizing the dimeric assembly (Jojoa-Cruz et al., 2018). The structure of two other OSCA channels was identified, for OSCA1.1 and OSCA3.1 (Zhang et al., 2018). They have structural similarity to the mammalian TMEM16 family proteins and the cone-shaped lipid Lyso-Phosphatidylcholine (LPC) can activate the channel suggests that the OSCA channels might directly sense force from lipid, akin to the well-studied MscL channel (Perozo et al., 2002). Recently, the isoform OSCA1.3 has been associated to PAMPs induced stomata closure (Thor et al., 2020). OSCA1.3 appears as a BOTRYTIS-INDUCED KINASE1 (BIK1) activated calcium permeable channel, suggesting that OSCA gene family can be also regulated by plant pathogen signaling pathway.

Members of two other MS ion channel families, the MscS-Like (MSL) channels and the Two-Pore K⁺ (TPK) channels, function on an organelle level. Several lines of evidence indicate that some of the MSL family proteins have MS channel activity. MSL9 and MSL10, localized in the plasma membrane in roots, provide MS channel activity, although electrophysiological studies suggest that MSL9 and MSL10 permeate Cl⁻ rather than Ca²⁺ (Peyronne et al., 2008). MSL10 has been very recently described to be a cell-swelling sensor (Basu et al., 2020). By using isoxaben (ISX) to soften the cell wall or plants transferred to a hypoosmotic solution which increases turgor that leads to cell swelling was shown that MSL10 is required for the calcium transient in a matter of seconds after the treatment. Cell swelling induced ROS accumulation after 30 minutes and programmed cell death after 3 days in MSL10 dependent manner. All these responses are regulated by the MSL10 phospho status as well (Basu et al., 2020). Another important role of MSL10 is its involvement in regulating cell death signaling which is

regulated by its N terminal domain (Basu et al., 2020, Veley et al., 2014) and this regulation is independent of its mechanosensitive properties (Veley et al., 2014).

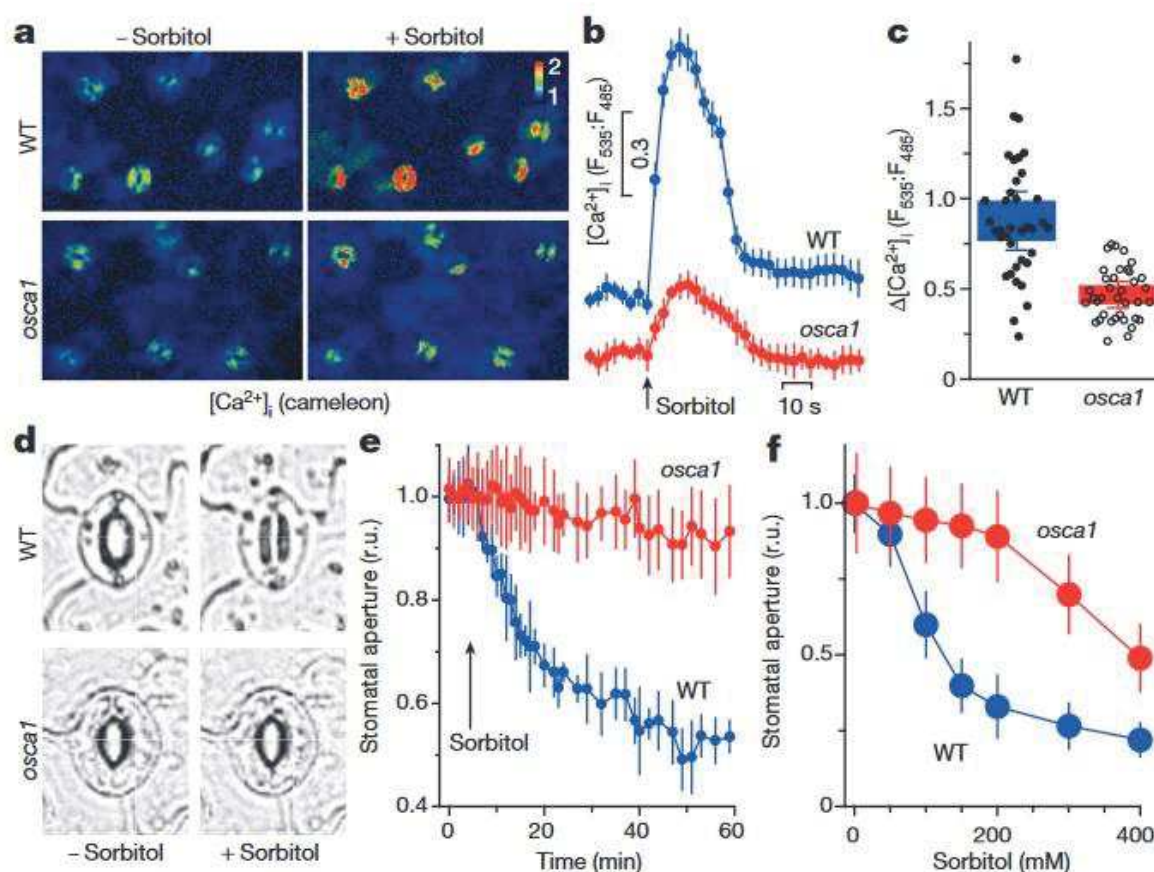


Figure 3: *osca1* plants have impaired guard cell osmotic stress signaling.

(A) Emission images (F535 nm and F485 nm) of epidermal strips from plants expressing YC3.6 were taken every 3 s, and ratiometric images (F535:F485) before and 20 s after addition of 200 mM sorbitol are shown. (B) The ratios were quantified from guard cell pairs in a (n55). (C) Peak ratio changes from experiments similar to a and bare shown (boxes represent the standard error (s.e.), error bars are s.d.; n 536;P,0.001).(D) Light images of epidermal strips were taken at varied intervals, and guard cell images before and 30 min after addition of 200 mM sorbitol are shown. (E) Changes in the width of stomatal pores in response to sorbitol from the same epidermal strips in d (mean 6 s.e.m.;n 55 stomata; two-way ANOVA,P,0.001; r.u., relative units) (F) Stomatal apertures are plotted as a function of applied sorbitol concentrations (mean 6 s.e.m.;n 580 for 150 mM and 200 mM, and 60 for others; two-way ANOVA,P,0.001). The apertures of wild type and *osca1* before the sorbitol treatment were 3.19mm and 3.05mm, respectively, and arbitrarily set to 1. Fang et al., 2014

MSL8 is a pollen specific MscS-like channel localized at the PM and endomembrane compartments and upon germination is mobilized to the pollen periphery (Hamilton et al., 2015). *Xenopus leavis* oocytes studies revealed that MSL8 indeed can produce mechanosensitive activities. MSL8 have important role in the survival rate of the mature pollen when experiencing osmotic downshift during rehydration. By using low concentration of PEG as mean to rehydrate pollen grains, *msl8-4* develops normally but losses viability due to hypo osmotic challenge of water entering the desiccated grain. During pollen germination, MSL8 can also maintain the optimal osmotic potential required to drive germination yet prevent lysis of the nascent pollen tube (Hamilton et al., 2015).

MSL2 and MSL3 are suggested to act as MS channels because both proteins can complement the lethality of an *E. coli* mutant lacking MscS under hypoosmotic conditions (Haswell and Meyerowitz, 2006). Both MSL2 and MSL3 proteins are also required to protect plastids from hypo-osmotic stress during normal plant growth. The *Arabidopsis msl2-1 msl3-1* double mutant has large, round plastids that lack dynamic tubular structures, known as stromules, on the surface. The leaf epidermal plastid phenotype of the *msl2-1 msl3-1* can be attributed to an abnormally high stromal osmolarity, leading to the influx of water, subsequent plastid swelling, and plastidic sensitivity to hypoosmotic shock. Thus, the stroma of leaf epidermal plastids has a lower osmotic potential (higher solute level) than the cytoplasm even under normal growth conditions and that MSL2 and MSL3 are required to relieve this hypo osmotic stress. (Veley et al., 2012).

Another emerging family of proteins with mechanosensitive properties is Defective Kernel 1 (DEK1). The DEK1 protein contains multiple predicted transmembrane (TM) spans interrupted by a loop, and an intracellular tail including a linker domain and a C-terminal domain showing similarity to animal calpains, a class of Ca^{2+} -dependent cysteine proteases (Lid et al., 2002). Structure-function studies have shown that the cytosolic CALPAIN domain of the protein can, alone, complement the embryo lethality of *dek1* mutants suggesting that this domain, which is removed from the rest of the protein by an autolytic cleavage event. They represent an active form of the DEK1 protein (Johnson et al., 2008). Calpains have been shown to act downstream of mechanosensitive channels such as PIEZO in animals (Li et al., 2014) and in planta Tran et al., 2017 showed that the TM domain of DEK1 is associated with Ca^{2+} dependent mechanoperception at the plasma membrane. DEK1 activity might lead to transient elevation of cytoplasmic Ca^{2+} concentration during mechanical stimulation, which is locally transduced by autolytic cleavage of the CALPAIN domain (Johnson et al., 2008). Based on the phenotype of lines with reduced DEK1 activity, this mechanotransduction pathway is likely

required for the maintenance of cell–cell contacts and epidermis integrity (Galetti et al., 2015), consistent with embryo lethality in loss of function DEK1 alleles (Tran et al., 2018)

1.3.3 Disruption in cell wall integrity

As stated above, osmotic stress can induce cell deformation. This has a direct effect on the continuum that exists between the cell wall and the plasma membrane and some plasma membrane anchored protein are suspected to perceive those changes. Wall associated kinases WAK1; leucine-rich repeat receptor-like kinases like FEI1, FEI2, MIK1; proteins linked to the cytoskeleton like Formin are few of the proteins able to perceive changes in the PM-CW-cytoskeleton complex (Rui and Dinenny, 2020). But amongst all of them, *Catharanthus roseus* RLK1-like kinases (CrRLK1Ls), are of particular interest and have been extensively studied for their role in cell wall sensing (more detailed description in Chapter 2). They contain a cytoplasmic serine/threonine kinase domain and two extracellular malectin-like domain that may bind to carbohydrates, as occurs in their animal counterparts (Schallus et al., 2010; Boisson-Dernier et al., 2011). THESEUS (THE) and FERONIA (FER) are some of the most studied CrRLK1Ls. Historically an EMS suppression screen on hypocotyl growth was made with PROCUST/Cellulose synthase 6 loss of function plant (PRC/CESA6). It was found that a mutation in THE1 attenuates drastically the hypocotyl elongation defect and ectopic lignin production of *pcr1*, suggesting a role in inhibiting cell expansion when cell wall defects are sensed (Hématy et al., 2007). FER, on the other hand, has much broader function profile and may serves as a signaling hub for many processes, including fertility, plant growth, hormone signaling, mechanosensing and plant defence (Liet al., 2016; Liao et al., 2017; Dunser et al., 2019). FER is required for the maintenance of a stable spatial distribution of cell expansion in the root elongation zone, and plays a key role in mediating mechanical signaling upstream of changes in cytosolic Ca²⁺ (Shih et al., 2014). In addition, Feng et al 2018 showed that salinity causes softening of the cell wall and that FER is necessary to sense these defects. When this function is disrupted in the *fer* mutant, root cells explode dramatically during growth recovery. Similar defects are observed in the *murus1* (*mur1*) mutant, which disrupts pectin cross-linking. Furthermore, *fer* cell-wall integrity defects can be rescued by treatment with calcium and borate, which also facilitate pectin cross-linking. Sensing of these salinity-induced wall defects might therefore be a direct consequence of physical interaction between the extracellular domain of FER and pectin. FER-dependent signaling elicits calcium transients that maintain cell-wall integrity during salt stress (Feng et al., 2018). Because both FER and THE have

malectin-like extracellular domains they may monitor cell wall status by binding directly to cell wall-derived carbohydrates or glycoproteins (Cheung and Wu, 2011). But recently a novel mechanism for cell wall sensing emerged, including cell wall extensin LRX and additional co-receptor LGG1 protein complexes with FER and corresponding peptides (Figure 4). This will be more detailed in chapter 3.

In summary, several hypotheses can hold the ground when talking about the plant osmo-perception. A mechanical cue of membrane or disruption events of the PM-CW continuum could contribute to the perception of the osmotic signal. What comes also from those studies is that a multitude of osmotic sensing mechanism coexists in cells. This is probably not surprising since the angle stone of water hydrostatic pressure has on plant physiology and development. One way to describe new molecular mechanism for osmotic sensing in plant is to use a bottom up approach starting from the activation of signal transduction. This is the strategy followed by the team and I will in the following section detail what is known about osmotic signal and cell secondary messengers.

1.4 Transduction of the osmotic stress signal

The basis for signal transduction is the transfer and integration of a certain signal through a cell. Here we will describe some crucial actors in the series of molecular events which take place between the osmotic signal perception and downstream cell responses. We have to note that indeed most of the below described secondary messengers are involved in many signalization and are really often interacting each other. In the following section, we will focus only on their part in the osmotic and osmotic-related signal transduction. For example, we will also consider response to salt as it induces osmotic stress to cell, in addition of its ionic effect.

1.4.1 pH

The proton activity (H^+) is an extremely important factor involved in cell signaling either directly or in cross talk with plant hormones or calcium (Gilroy and Trewavas, 1994; Ward et al., 1995; Blatt and Grabov, 1997; Roos, 2000; Felle, 2001). Therefore, the cells tightly control H^+ concentration especially between the two sides of the plasma membrane (Felle, 1987). Salt overly sensitive (SOS1) gene has been suggested to function as a Na^+/H^+ antiporter located at the plasma membrane of plant cells that can regulate pH. Guo et al., 2009 described that *sos1* mutants showed net H^+ efflux and intracellular alkalization in the meristem cells. Upon NaCl treatment, the internal pH gets to higher values in WT and *sos1* plants. Thus the SOS1 can mediate the pH upon salt treatment (Guo et al., 2009). Regarding the impact of osmotic stress on cell pH, only a limited number of studies are available. They suggest that osmotic signal

has a little effect on pH either in the cell apoplast or cytoplasm (Gao et al., 2003).

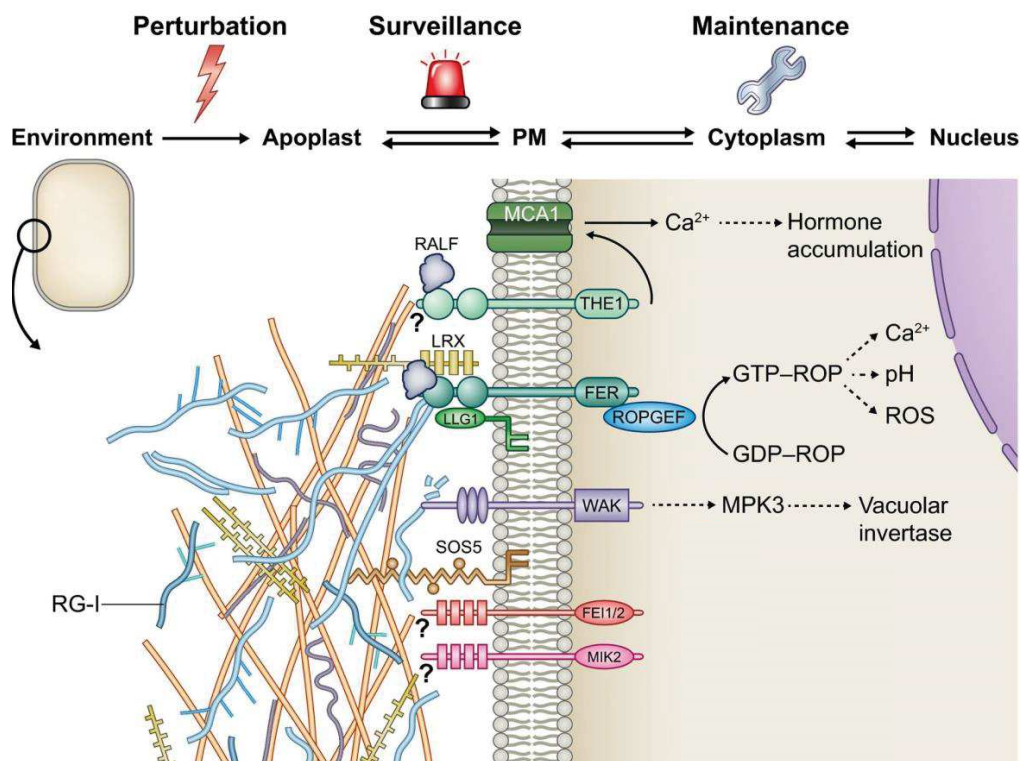


Figure 4: **Cell wall surveillance and maintenance.** Cells can sense genetic or environmental perturbations to the wall and sends the signal across the plasma membrane (PM) to the cytoplasm, triggering downstream events that eventually feedback to compensate any changes in the apoplast. Arrows at the top of the figure represent the flow of information. Major components of the primary cell wall are depicted in the apoplast that faces the environment. Different classes of membrane proteins that are hypothesized to be CWI sensors are shown. Question marks in the apoplast indicate that the identity of the ligand is uncertain. Adapted from Rui and Dinneny, 2020.

1.4.2 Calcium ion

Calcium (Ca^{2+}) is ubiquitous secondary messenger that can regulate many different cellular functions. Over 15 years ago, Berridge and co-workers proposed a framework, known as the “ Ca^{2+} signaling toolkit”, around which to build our current understanding of the operation of Ca^{2+} based signaling (Berridge and Bootman, 2000; Berridge et al., 2003). Specificity of Ca^{2+} cyt signaling is determined by the amplitude and duration (and possible oscillation) of the Ca^{2+} cyt increase, often referred to as the “signature” (McAinsh and Pittman, 2009), that is elicited by the stimulus. This signature would be driven by the opening of PM and endomembrane Ca^{2+} -permeable channels and terminated by the activity of Ca^{2+} efflux transporters in those membranes. Decoding the cytoplasmic Ca signature will be effected by specific Ca^{2+} -binding proteins. Calmodulins (CaMs) and Calmodulin-like proteins (CMLs) are encoded by multi-gene families in plants. CaMs modulate transcription by binding to Calmodulin-binding Transcription Activators (CAMTAs) (Virdi et al., 2015). Other multi-gene families are also evident for Ca^{2+} -Dependent Protein Kinases (CPKs) and Calcineurin-B Like proteins (CBLs). The latter target CBL-Interacting Protein Kinases (CIPKs) to effect cellular responses (Thoday-Kennedy et al., 2015).

Cytosolic calcium (Ca^{2+} cyt) is central to the responses in osmotic stress conditions (Carroll et al., 1998). Hyperosmotic stress induces a rapid rise in intracellular Ca^{2+} concentrations in plants, and this Ca^{2+} response may reflect the activities of osmosensory components. When the osmolarities of the stimuli were increased (300-1800mOsmol), the amplitudes of the rapid Ca^{2+} responses increase dramatically. This response is regulated by a plastidial K^+ exchange antiporter (KEA)1/2/3 considering that *kea1-2kea2-2* double mutants and *kea3-1* mutants showed reduced osmotic-induced Ca^{2+} responses (Stephan et al., 2016). In the same study the authors described another interesting feature of the Ca signaling. Under constant hyperosmotic stimulation, the Ca^{2+} response returns to baseline levels. This return suggests that negative feedback mechanisms must exist to inactivate sensory components, including closing Ca^{2+} channels and removing cytosolic Ca^{2+} . This sensory adaptation contributes to the ‘priming’ where sensitivity of the sensory components is increased after a previous experience of hyperosmotic stress.(Sani et al., 2013, Stephan et al., 2016).

Another very important feature of the Ca^{2+} is its involvement in hydrotropism responses of the root. Hydrotropism is the ability of an organ to grow toward the higher water potential. Forward genetics approaches revealed that Mizu-Kussey 1 and 2 (MIZ1 and MIZ2) are needed for root hydrotropism (Kobayashi et al., 2007; Miyazava et al., 2009). Root bending in response to

hydrostimulation requires long-distance cytosolic Ca^{2+} mobilization from the root cap to the elongation zone. This is mediated by the interaction of MIZ1 with the type 2A Ca^{2+} -ATPase isoform ECA1, an ER-localized Ca^{2+} efflux carrier (Shkolnik et al., 2018). But how MIZ1 or MIZ2 get activated by osmotic signal is still a mystery.

1.4.3 Lipids

Signaling lipids are low abundant (<1% of total lipids) and have a fast turnover. They include a wide range of lipid classes, such as lysophospholipid, fatty acid, phosphatidic acid, inositol phosphate, diacylglycerol, oxylipin, sphingolipid and N -acylethanolamine (Wang, 2004; Wang et al., 2006; Kang et al., 2008; Munnik and Testerink, 2009; Kilaru et al., 2009; Markham et al., 2013). These lipids are usually quickly synthesized from pre-existing membrane lipids or biosynthetic intermediates of membrane lipids.

Phosphatidic acid (PA) is one of the best describe signaling lipid for its role in response to an osmotic signal. Outside of the ER (endoplasmic reticulum), PA is a hydrolyzed product of Phosphatidylcholine (PC) by Phospholipase D (PLD), but can also be produce by Diacylglycerol kinase (DGK) from diacylglycerol (DAG). PA and PLD are needed to mediate many plant response to their environment including hyperosmotic and salt response (Hong et al., 2008 ;Munnik et al., 2000; Yu et al., 2010), response to temperature (Ruelland et al., 2002; Arisz et al., 2013) and abscisic acid (ABA) treatment (Katagiri et al., 2005; Uraji et al., 2012). They are involved in nutrient sensing such as nitrogen and increased root surface enables plants to uptake and utilize water more efficiently during hyperosmotic stress. PA might function as a membrane localized signal to recruit specific target proteins, which upon binding change their activity. For example, PA has been reported to promote stomata closure by binding and inhibiting the protein phosphatase ABI1, a negative regulator of the ABA response (Zhang et al., 2004). PA is found to bind to AtrbohD and AtrbohF NADPH oxidases, and the PA binding motifs in RbohD and RbohF have been identified near the N terminus (Zhang et al., 2009). The mutated RbohD, which lacks PA binding, failed to mediate PA-induced ROS elevation and stomatal closure and was compromised. PA is also a crucial modulator of PIN2 activity and auxin redistribution in response to salt stress, in other words during salt stress PA maintains PIN2 at the PM. Under salt stress, loss of phospholipase D function impaired auxin redistribution and resulted in markedly reduced primary root growth due to lower levels of PA. These effects were reversed by adding exogenous PA (Wang et al., 2019)

Despite the growing number of confirmed PA target proteins in plants, no consensus PA-binding motif has been identified. Hence, the 'electrostatic/hydrogen-bond switch' model, might

explains the molecular basis of PA binding based on the unique properties of PA (Kooijman and Testerink, 2010, Noack and Jaillais, 2019). The precise mechanism by which a specific PLD is activated under osmotic stress conditions remains to be defined. Available results suggest that the regulation of the PLD family is complex, involving multiple effectors, including Ca^{2+} , PIP2, G proteins, and positive and negative feedback loops.

1.4.4 Osmotic stress kinases

Protein phosphorylation is such a central theme in cell signaling that its involvement in osmotic stress adaptation was studied a while ago. Hyperosmotic stress can lead to increased mRNA levels (Kilian et al., 2007). As a primary response to osmotic stress, however, there must be a more direct and rapid signaling before activation of gene transcription and that signaling evidently involves kinase activity: osmotic stress increases the enzymatic activity of some kinases, which are referred to as osmotic stress-activated kinases.

Using in-gel kinase assays from Arabidopsis seedlings extracts and tobacco cell suspensions, several protein kinases were identified that are activated by osmotic stress in plants (Mikołajczyk et al., 2000; Boudsocq et al., 2004).

SnRK2 is a plant specific protein kinase family related to yeast osmotic kinase SNF1; the SnRK2 family has 10 members (SnRK2.1-2.10) in Arabidopsis and 10 members (SAPK1-10) in *Oryza sativa* (Hrabak et al., 2003). The pull-down fraction from Arabidopsis crude extract obtained with anti-SnRK2 antibody contained osmotic stress-activated kinases Mikołajczyk et al., 2000). In Arabidopsis protoplasts all SnRK2 except SnRK2.9 are activated by mannitol and NaCl (Boudsocq et al., 2004). Decuple mutants of all 10 SnrK lack osmotic stress activated bands and grows poorly on hyperosmotic media but grows normally on half-strength Murashige-Skoog (MS) medium (Fujii et al., 2011). But, single mutants *snrk2.7*, *snrk2.8*, or *snrk2.2/3/6* triple or *snrk2.1/4/5/7/8/9/10* septuple show similar drought soil tolerance compared to WT (Mizoguchi et al., 2010). This demonstrate a high degree of redundancy of osmotically induce SnRK2.

Recent studies show that SnRK2 might be regulated by upstream Raf-like protein kinases, which can phosphorylate and activate SnRk2. The RAFs are likely also important for osmoregulation during growth and development, as the osmotic kinase (Ok) -null and OK-quinndec (*raf16*; *raf40*; *RAF24*^{Δ10}; *raf18*; *raf20*; *raf35*; *RAF42*^{Δ6}; *raf2/edr1*; *raf3*; *raf4*; *raf5/sis8*; *raf7*; *raf8*; *raf9*; *raf10*) mutants show strong growth and developmental defects (Zhen et al., 2020).

Other osmotic stress-activated protein kinases in addition to SnRK2s have been reported. It

has been previously well described the mechanisms of the osmotic signaling in yeast and mammalian systems. In *Saccharomyces cerevisiae*, upon osmotic stress, the mitogen-activated protein kinase (MAPK) is activated by Src-homology 3 (Sh3)-domain containing protein and two histidine kinase. This activation results in increased osmolyte synthesis (Kenneth and Davenport, 1998). Several MAPKs in plants are activated by hyperosmotic stress such as MAPK4 and MAPK 6 (Ichimura et al., 2000) but their complete involvement in osmotic signaling is yet to be elucidated.

1.4.5 ROS

Reactive oxygen species are at the cross road of plant signaling and a substantial number of papers have established its role during osmotic signaling in plant cell.

The traditional notion that ROS are toxic by-products of plant metabolism has changed. Substantial experimental data are available assuring that ROS are highly controlled signaling molecules able to transfer the environmental signals. To utilize ROS as signaling molecules, non-toxic levels must be maintained in a delicate balance between ROS production, involving ROS-producing enzymes and the metabolic counter-process involving ROS-scavenging pathways (Mittler et al., 2004).

During normal metabolic activity, and also as a result of various environmental stresses, the oxygen O_2 is capable of giving rise to dangerous reactive states such as free radicals (Polidoros et al., 2004; Phaniendra et al., 2015). Reactive oxygen intermediates may result from the excitation of O_2 to form singlet oxygen (O_2^1 ; Triantaphylidès and Havaux, 2009) or from the transfer of one, two, or three electrons to O_2 to form, respectively, a superoxide radical ($O_2^{\cdot-}$), H_2O_2 or a hydroxyl radical ($\cdot OH$; Mittler, 2002).

In vivo ROS production

During normal metabolic processes, but also during abiotic and biotic stresses the cell accumulates ROS (Bhattacharjee, 2005). Mittler reviewed several sources for production of ROS in plant cells, including, in addition to photosynthetic (ROS can be produced at photosystem I (PSI) as well as at PSII) and respiratory electron transport chains (1 to 5% of the oxygen taken up by isolated mitochondria is used in ROS production), photorespiration, amine oxidase, and cell wall-bound peroxidases. But one of the most important families of enzyme for ROS signaling is the Rbohs.

1.4.6 RBOH a major actor of ROS signaling

Plant Rbohs have cytosolic FAD- and NADPH-binding domains in the C-terminal region, and six conserved transmembrane-spanning domains that correspond to those in mammalian NOX (Kobayashi et al., 2007; Lin et al., 2009; Glyan'ko and Ischenko, 2010; Proels et al., 2010; Kimura et al., 2012). There are 10 members in Arabidopsis (A-J) (Torres and Dangl, 2005). Unlike the mammalian counterpart, the proteins p47phox, p67phox, p40phox, and p22phox that regulate NOX proteins are missing in plants. Instead plants Rbohs have a cytosolic N-terminal extension that is important for activity regulation. It contains two Ca²⁺ binding EF-hand motifs, PA binding motif (Li et al., 2019), small GTPase binding site and phosphorylation target sites (Kobayashi et al., 2007; Oda et al., 2010; Kimura et al., 2012; Drerup et al., 2013). The Arabidopsis double mutant *rbohDxrbohF* in the dSpm insertion mutagenesis system produces significantly decreased ROS against infection with avirulent *Pseudomonas syringae* pv *tomato* DC3000 or *Hyaloperonospora parasitica* (Torres et al., 2002). *rbohDxrbohF* also show decreased ROS production in response to abscisic acid (ABA) and is impaired in ABA-activated stomatal closure (Kwak et al., 2003). Nb *RbohA*/Nb *RbohB*-silenced *Nicotiana benthamiana* plants show a reduced oxidative burst and reduced disease resistance to *Phytophthora infestans* (Yoshioka et al., 2003). ROS produce by Rboh is also crucial for developpement. Especially, it was demonstrated to participate to lateral root developpement (Orman-Ligeza et al., 2016) and it is also important for tip growing cell. Indeed, Arabidopsis *rbohC* has a defect in Ca²⁺ uptake and ROS accumulation during root hair formation (Foreman et al., 2003). All these reports suggest that Rboh is a key regulator of ROS production and displays pleiotropic functions in plants.

ROS and osmotic signaling

ROS, which accumulate in the frame of tens of minutes after osmotic stress, represent a key second messenger during hyperosmotic signaling (Leshem et al., 2007). This signaling molecule is needed to activate cellular, developmental and physiological responses to osmotic signal. For instance, Rejeb et al., 2015 reported that H₂O₂ induced proline accumulation. The proline is the first amino acid to accumulate in plants subjected to water deficit stress. But, ROS was also associated to fast organ response. Regulation of root water conductivity is a fast response that prevents water loose during salt or osmotic stress. Interestingly, it was demonstrated that root pretreated with catalase that detoxify H₂O₂ to H₂O, cannot diminish their water conductivity in response to osmotic stress. Oppositely, H₂O₂ can inhibit the root water permeability when exogenously added in the system. Boursiac et al., 2008 have studied the

potential role of H_2O_2 on the root water channels, the aquaporins, and found that H_2O_2 don't gate aquaporin through direct oxidative mechanisms nor regulate aquaporin transcription or protein abundance but instead act via signaling mechanisms. GFP tag of PIP2-1 and PIP1-2 express in arabidopsis show that salt stress induces PIP2.1- internalization (Boursiac et al., 2005). Indeed, most of plasma membrane localized protein undergoes constant cycling. This mechanism that can be totally independent of protein synthesis or degradation, specifically add or remove protein form the PM depending of external stimulus (explained in Chapter 1-3.2.2). Later, it was show that both endocytosis and exocytosis is induced in response to salt or osmotic stress that acts on the speed of PIP2.1 and PIP1.2 cycling (Luu et al., 2012). In addition, it was shown that PIP forms domains in membrane (Li et al., 2012). After cell stimulation with salt, those domains start to diffuse and PIP2.1 gets endocytosed by a clathrin dependent and independent pathway (Li et al., 2012). Interestingly, if root are pretreated with catalase, salt induce PIP2;1 internalization can be substantially reduce (Figure 5) (Boursiac et al., 2008). Exogenous addition of H_2O_2 in the media acts on PIP2.1 dynamic in cell in a very similar manner than salt stress, especially PIP2.1 gets endocytosed (Wudick et al, 2015).

These sets of results show that ROS are acting upstream of PIP2.1 endocytosis and most likely to regulate root water conductivity. Therefore ROS is nicely associated with a very specific and fast cellular responses the endocytosis of PIP2.1. The lab later used this response to better characterize the ROS producing machinery.

In this line, Martiniere et al., 2019 describe that indeed ROS are produced in hyperosmotic conditions and this can enhance the FM4-64 labeled BFA bodies upon sorbitol treatment. ROS triggered protein internalization effect is selective for certain cargo molecules. As mentioned above some aquaporins can be internalized to avoid water loss but other PM resident proteins aren't (such as AHA2 H^+ -ATPase). This indicates for certain specificity.

The second objective of this work was to identify the ROS producing machinery in response to osmotic stimulation. As expected, the conventional enzymatic pathway by the Rboh, RbohF and RbohD, is necessary for ROS accumulation but also to induce PIP2;1 internalization. But more surprisingly, it was shown that this mechanism is not sufficient to explain all the ROS produce in response to osmotic signal. .An additional pathway, involving apoplastic ascorbate and transition metal, exists. ROS is arising from a non-enzymatic activity, as well (Figure 6).

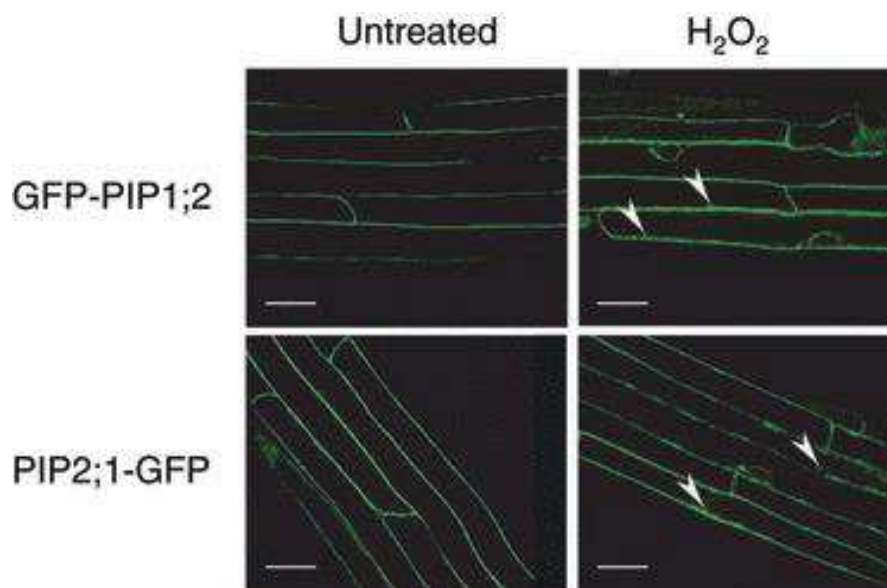


Figure 5: **Effects of hydrogen peroxide (H_2O_2) on the subcellular localization of plasma membrane intrinsic protein (PIP) aquaporins in root epidermal cells.** Roots of plants expressing GFP-PIP1;2 or PIP2;1-GFP were incubated for 15 min in the absence (Untreated) or in the presence of 2mM H_2O_2 (H_2O_2), and images of epidermal cells were taken at 15 min (GFP-PIP1;2) or 30 min (PIP2;1-GFP) from the root apex. Arrowheads indicate the labelling of small intracellular spherical bodies. Scale bar: 20 μ m. Boursiac et al., 2008.

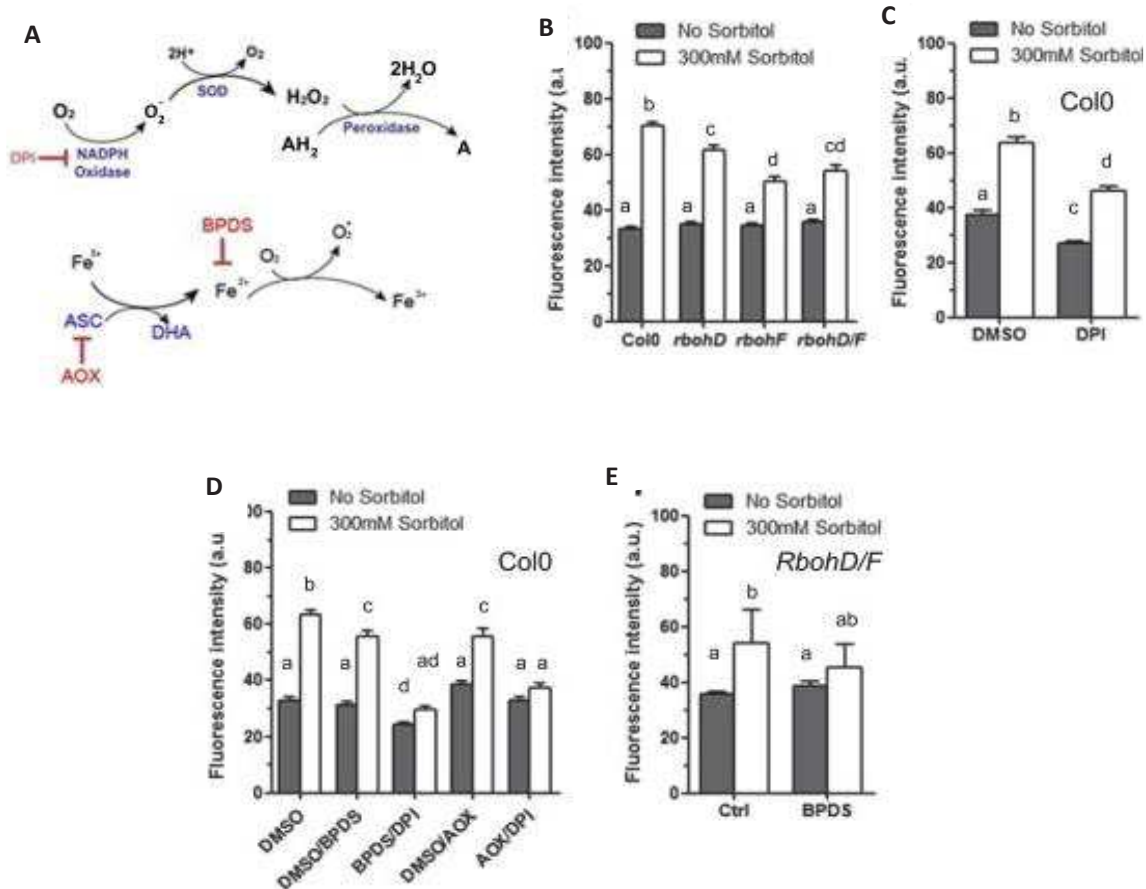
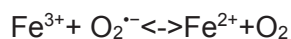


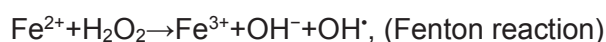
Figure 6: **ROS accumulation in root cells after a sorbitol treatment.** (A), Schematic representation of ROS production as mediated by NADPH oxidases and by the putative Asc/Fe pathway. (B) Quantification of DHE fluorescence in Col-0, *rbohD*, *rbohF*, and *rbohD/F* plants subjected or not to the sorbitol treatment. Effects on DHE fluorescence of a 30-min pretreatment, with either dimethyl sulfoxide (DMSO) or DPI, before incubation for 15 min with 5 μ M DHE followed by a mock (no sorbitol) or 300 mM sorbitol treatment on Col-0 (C). (D) Effects on DHE fluorescence of a 30-min pretreatment with BPDS (Fe^{2+} chelation) or AOX (ascorbate depletion), alone or in combination with DPI (Rboh inhibition). (E) Effect of a 30-min pretreatment with BPDS on DHE fluorescence in the *rbohD/F* double mutant. Martiniere et al., 2019

The non-enzymatic way of producing ROS can arise in aerobic solutions of ferrous salts by the iron catalyzed Haber-Weiss process:

First, Fe^{2+} in presence of O_2 can give Fe^{3+} and $\text{O}_2^{\cdot-}$ in the first part of the Fenton reaction. Note that this reaction is fully reversible.



Later on the reaction continues as it follows:



Martiniere et al 2019 described that Fe^{2+} is indeed involved in the osmotically induced ROS accumulation by using bathophenanthrolinedisulfonic acid (BPDS). BPDS is Fe^{2+} chelator that depletes free Fe^{2+} in the cell apoplast. More importantly, if BPDS is used in combination with an inhibitor of Rboh activity or in *rbohDxrbohF* background all the ROS produce in response to osmotic stimulation is suppress. This show that Fe^{2+} chelation and RBOHs activity are the two pathways needed to produce ROS.

Since the lifetime of the reduced iron form is very fast and because of its high reactivity, a reducing agent must be active during osmotic signaling. They hypothesized that ascorbate (Asc) might be involved because is the most abundant, low molecular weight antioxidant that has a key role in regulation of oxidative stress in plant. By using ascorbate oxidase 1 (AOX1, which depletes the apoplastic pool of Asc) or a mutant *vtc2.4* (hat has 70% less ascorbate levels in leaves) the levels of osmotically induced ROS were diminished. In addition, root treated with Asc shows an induction of ROS. Thus, this suggested that Asc is a positive regulator of ROS during hyperosmotic shock, probably by its action on Fe^{3+} reduction effect.

The Rboh and the Iron/Asc pathway both contribute to bulk membrane internalization. The FM4-64 labeled BFA bodies were more numerous upon hyperosmotic treatment and in plants treated with inhibitors of the both ROS producing pathways, a decrease of the FM4-64 labeled BFA bodies was noted (Martiniere et al., 2019). This indicated that the ROS produced by these 2 mechanisms activate the endocytosis machinery (Maritiniere et al., 2019, Wudick et al., 2015).

Because, AOX enzyme is strictly acting outside of the cell and apoplast is mostly an oxidized environment, it is suspected that cytoplasmic reductant power is transfer to the apoplast upon

osmotic stimulation. One possibility is that Asc is transfer through cell PM. In line with this, Grillet et al., 2014 described that indeed an Asc efflux system is present during iron uptake in the embryo. Another alternative is a cytoplasmic reducing power may be transferred through the cell membrane, by cytochrome proteins for instance, to regenerate the apoplastic dehydroascorbate (DHA) pool. In tobacco, the balance between the Asc and DHA is pretty important as the missregulation of AOX can decrease plant pathogen responses and alter auxin signaling (Pignocchi et al., 2006). As stated above, many scenarios are possible in line of the non-enzymatic ROS production but the complete molecular mechanisms are still largely unknown.

Out of this work, ROS producing machinery has been describe but it is not really clear how it gets activated. For the Asc/iron pathway, a molecular mechanism is difficult to hypothesis since it's not really known how reductant power from the cytoplasm gets transferred to the apoplast. For example, it could be through a direct efflux of Asc but also through electron transfer to regenerate DHA. At the opposite, the RBOH activation is way better characterized. We will detail in the next section some example of these regulations.

1.4.6.1 Direct and indirect regulation by calcium

Since Rbohs have Ca²⁺-binding EF-hand motifs in their N-terminal regions, Ca²⁺-was thought to be important for their regulation. Mutation in EF hands, shown by Ogasawara et al., 2008, results in decrease of ROS production. Structural and biochemical analysis showed that OsRbohB has two EF-hand-like motifs, but Ca²⁺-only binds to the first EF-hand motif (Oda et al., 2010).

In addition to direct calcium binding, Rboh was demonstrated to be regulated by phosphorylation. Two types of kinase activated by calcium are known to phosphorylate Rbohs. CPKs are important regulators of Rboh in terms of the Ca signaling. They are plant proteins that directly bind calcium ions through interactions with alpha-helices (Wernimont et al., 2010) before phosphorylating substrates involved in metabolism, osmosis, hormone response and stress signaling pathways. CPK4, CPK5, CPK6 and CPK11 are positive regulators of the PAMP-induced ROS burst (Boudsocq et al., 2010). CPK5 can phosphorylate RbohD and to regulate its activity (Dubiella et al., 2013). Yeast 2 hybrid study was used, that identified CIPK26 as a specific interactor of the RbohF N-terminal region and it's its negative regulator (Kimura et al., 2012). CIPK26, when coexpressed with, the Ca sensors, CBL1 or CBL9 enhances ROS production by RbohF in HEK293 cells, suggesting that the CBL1/9–CIPK26

complex has a positive role in RbohF-mediated ROS production (Drerup et al., 2013) (Figure 7).

1.4.6.2 RBOH regulation by BOTRYTIS-INDUCED KINASE1 (BIK1)

The last type of kinase describe to regulate Rboh comes from extensive studies over the years have been made on ROS produced as a result of an immune response (Kaku et al., 2006, Chinchilla et al., 2006, Zipfel et al., 2006, Miya et al., 2007, Wan et al., 2008,). A major class of the plant immune receptors is the cell surface-localized pattern-recognition receptors (PRRs) that detect conserved microbial elicitors. FLS2 is well known PRR receptor that can recognize flagelin22 (flg22) and with this have very important role in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). The leucine rich repeat (LRR) domain of FLS2 perceives flg22 and rapidly recruits a LRR receptor-like kinase co-receptor called BAK1 (Chinchilla et al., 2007, Heese et al., 2007, Schulze et al., 2010). Intermolecular interactions between the FLS2 LRR domain, flg22, and the BAK1 LRR domain showed the activation of the PRR complex (Sun et al., 2013). In the PTI response, BAK1 associates with BIK1 which is downstream of BAK1. Li et al., 2014 described that BIK1 can directly phosphorylate RbohD upon flg22 perception. Immunoprecipitation studies by Kadota et al., 2014 showed the interaction between BIK1 and RbohD in presence of both flg22 and elf18 (another type of PTI elicitor) in absence of Ca signaling. In vitro quantitative phosphoproteomics showed some specific BIK1 mediated phosphorylation sites (S39, S339, and S343) and CPK-mediated phosphorylation sites (S133 and S163). BIK1-mediated phosphorylation also occurred in the absence of Ca²⁺ or in the *cpk5* mutant, showing that BIK1-mediated phosphorylations at S39 and S343 are independent of Ca²⁺-based regulations. The phosphorylation of RbohD, since it is happening of the N-terminus, where the EF-hand residues are, might contribute to Ca binding to those residues with a higher affinity. The phosphorylation of RbohD is not sufficient to trigger the PAMP-induced ROS, Ca²⁺ and CPKs are still required ultimately to induce a full ROS burst because *bik1* mutants have low Ca burst phenotype (Li et al., 2014) .This is why the BIK1 phosphorylation might be a “priming system” for the Ca induced ROS (Figure 7).

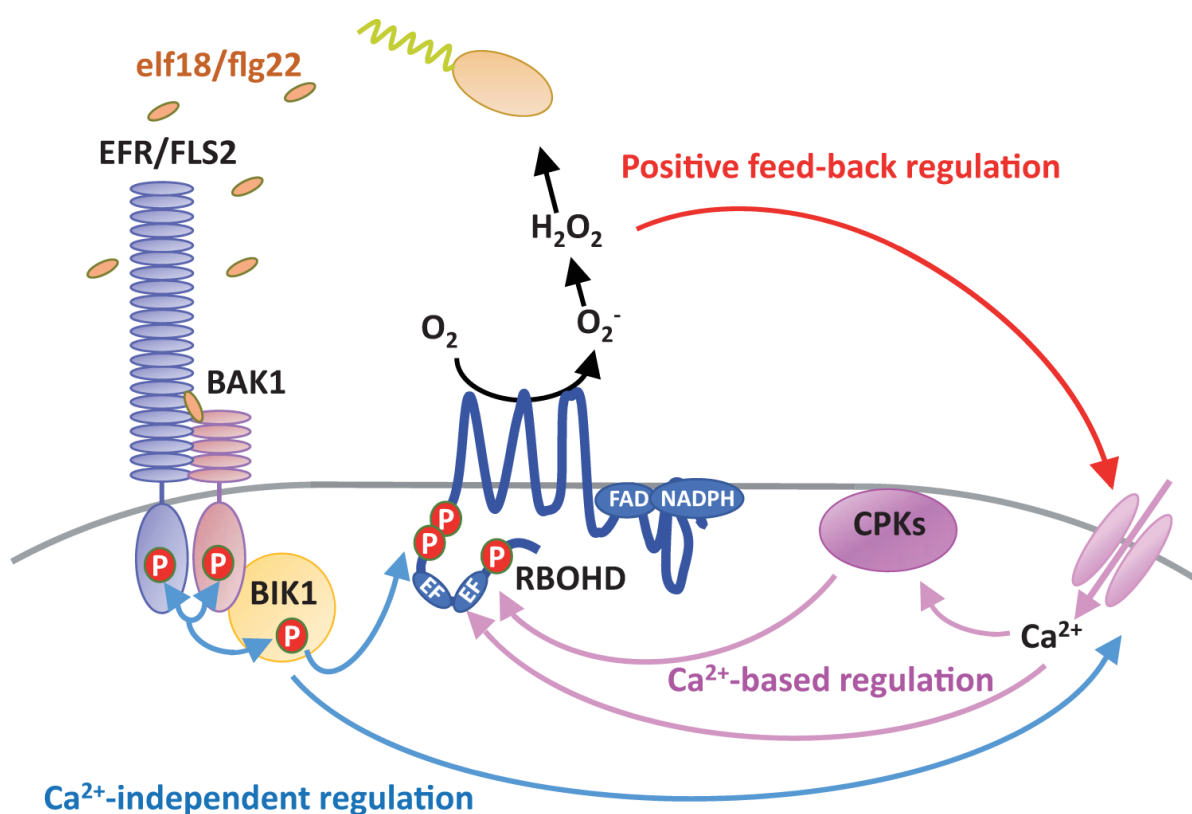


Figure 7: **A two-step activation model for RbohD.** Upon PAMP perception, PRRs, such as EFR and FLS2, and their co-receptor BAK1 directly phosphorylate and activate BIK1. Phosphorylated BIK1 has a higher binding affinity for RbohD and phosphorylates it on some specific sites. BIK1-mediated phosphorylation may 'prime' the Ca²⁺-based regulation of RbohD by inducing conformational changes that could lead to increased Ca²⁺ binding affinity for EF-hand motifs and/or increased accessibility for CPK-mediated phosphorylation. At the same time, PRRs together with BIK1 also activate Ca²⁺ channel(s) and induce Ca²⁺ influx directly or indirectly. This leads to Ca²⁺ binding to an EF-hand motif in RbohD and also activation of CPKs, which in turn phosphorylates RbohD. The produced H₂O₂ itself may trigger further activation of Ca²⁺ channel(s), leading to the full activation of Ca²⁺ signaling and Ca²⁺-based regulation of RbohD. Kadota et al., 2014.

1.4.6.3 Membrane domains regulation on Rboh

The lateral organization of the cell membrane critically influences the kinetic properties of membrane proteins. Lipid rafts or detergent resistant membranes (DRMs) are emerging as a crucial membrane feature for protein regulation. A massive plant proteomics analysis identified NtRbohD and its regulator, the small G protein Rac5, involved in the signal transduction triggered by the fungal elicitor cryptogein, in DRMs. This has been previously immunologically detected in tobacco DRMs (Morel et al., 2006; Mongrand et al., 2004). Hao et al 2014 plant cell showed that under several abiotic related stimuli (NaCl and ABA treatment) RbohD reassemble into domains and then gets internalized. Variable-angle total internal reflection fluorescence microscopy analysis showed that disruption in the microdomains by a sterol inhibiting drug (m β CD) leads to increased RbohD density. Knock down in a possible scaffold membrane protein Flot1 decreases the diffusion coefficient of RbohD. The pool of RbohD clusters forms homodimers and flg22 and ABA treatment increase this homodimerization (Hao et al., 2014)

1.4.6.4 Small GTPase regulation of RBOH activity

In mammalian cells, the regulation of RAC small GTP-ase over NOX has been intensively studied. NOX2, the best-characterized mammalian NOX comprises of the flavocytochrome b558, which is a heme-binding heterodimer composed of a large (gp91phox) and a small (p22phox) subunit. The gp91phox subunit has 6 transmembrane regions in its N terminus, whereas its C-terminal portion contains the flavin-adenine dinucleotide (FAD) and NADPH binding domains that are essential for activity (Wallach et al., 1996; Nauseef et al., 2004). Oxidase activation is controlled by the recruitment of regulatory proteins to the flavocytochrome, including the p40phox, p47phox and p67phox, of which p47phox and p67phox are essential for activity (Levy et al., 1990; Heyworth et al., 1991). A human X-linked genetic disorder, chronic granulomatous disease (CGD) results from >400 different mutations that have been identified in members of the oxidase complex, including gp91phox, p22phox, p67phox, p47phox, or Rac2. This leads to a lack of proper complex assembly or to an inactive gp91phox subunit (Heyworth et al., 2003). Rac2 is small GTPase crucial for the normal functioning of NOX2 because it acts in the recruitment of p67phox, which associates with p47phox and with the cytochrome (Dang et al., 2002) Also a direct binding of Rac2 to the flavocytochrome has been implicated in the initial steps of the electron transfer reaction

(Diebold et al., 2001) (Figure 8).

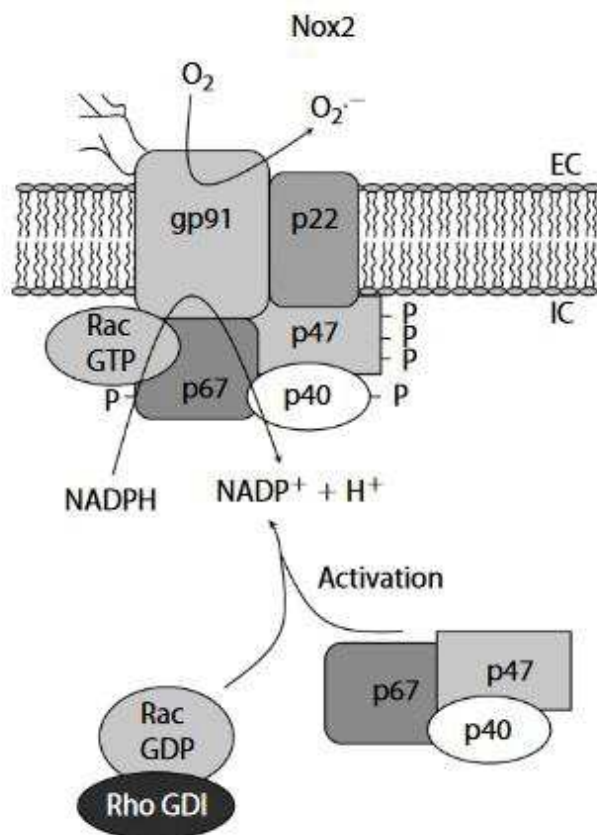


Figure 8: **Activated complexes of multicomponent animal NOX2-based NADPH oxidase.** The Nox2 (gp91phox) flavocytochromes form heterodimeric complexes with a common p22phoxchain. NOX2 regulated by homologous organizer (p47phox) and activator(p67phox) proteins, and require GTP-bound Rac. The cytosolic subunits of Nox2 (p47phox,p67phox and p40phox) are preassembled in the cytosol and translocate to the flavocytochrome upon activation. In resting cells, Rac is found in a GDP-bound state stabilized by RhoGDI. When activated NOX2 produce superoxide anions. Nox1 is localized to the plasma membrane of colon epithelial cells and produces superoxide into the extracellular space, whereas Nox2 is assembled and activated on phagosomes of phagocytic cells EC- extracellular, IC- intracellular. Adapted from Rada et al., 2008.

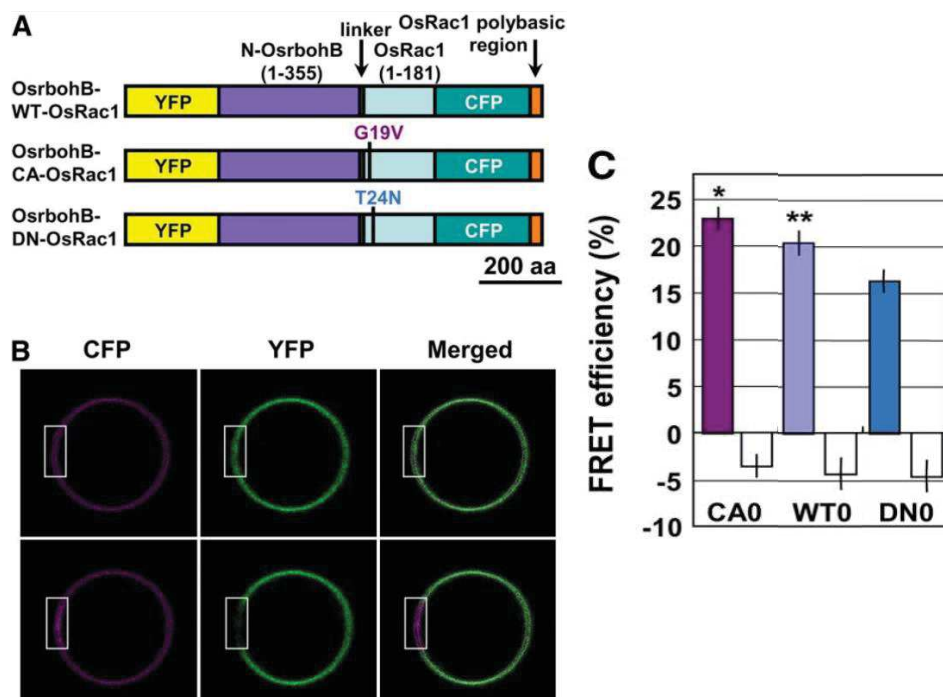


Figure 9: In Vivo FRET Analysis in Transiently Transformed Rice Protoplasts. (A) Schematic representation of Os Rac1-Rboh intramolecular FRET constructs used for transient assays. (B) CFP and YFP fluorescence of rice protoplast expressing Os Rac1-Rboh FRET construct before (top panels) and after (bottom panels) YFP photobleaching. The region marked in white was used for YFP photobleaching. (C) Colored bars indicate calculated mean FRET efficiencies of FRET constructs containing CA-OsRac1 (CA), wild type-OsRac1 (WT), or DN-OsRac1 (DN). Open bars indicate mean background FRET efficiencies, which represent the percentages of change in CFP fluorescence caused by the imaging process, without acceptor photobleaching (Bhat et al., 2004). Single asterisk and double asterisks indicate significant difference from DN0 by *t* test at $P < 0.005$ and $P < 0.05$, respectively (Oda et al., 2010).

RAC homologues in plants exist and they have been widely characterized as one of the potential regulators of Rboh activity. The rice RAC1 was described to be a positive regulator of OsRbohB (Oda et al., 2010). OsRAC1 interacts with the N-ter of OsRbohB and point mutation in RAC1 conforming it to constitutively active protein leads to ROS accumulation in tobacco and a dominant-negative (DN) form of RAC1 causes reduction of ROS levels (Suharsono et al., 2002), Structural analyses of OsRbohB, coupled with in vitro binding and NMR (nuclear magnetic resonance) titration assays and in vivo fluorescence resonance energy transfer (FRET) microscopy from rice protoplasts, showed direct interaction between RAC1 and RbohB (Figure 9). OsRAC1 binds the coiled-coil region that is made from the homodimerization of the N terminal region. This homodimerization is required for the RAC1 mediated regulation over OsRbohB (Kosami et al., 2014, Oda et al., 2010, Wong et al., 2007). The constitutive active ROP6-CA has defaults in the ROS distribution in the RH. In the tip growing cells such as RH the highest accumulation of ROS is on the tip of the cell but constantly activated ROP6 has ROS distributed throughout the cell and losses the tip gradient (Sorek et al., 2010). This in return has an effect on the RH morphology; RH are swollen. ROP6-CA also has blocked FM4-64 endocytosis. This is in line with the results showing that ectopic expression of RAC10 can modify membrane cycling (Bloch et al., 2005)

Investigating ROP11 and RbohF it was revealed that ROP11-CA specifically interacts with RbohF, and the amino acids Leu336 and Leu337 in RbohF were key sites for its interaction with ROP11 CA-. Mutated RbohF don't bind to CA-ROP11, suggesting that ROP11 modulates ROS production by regulating RbohF activity during root hair development (Yan et al., 2016).

In conclusion, all the above examples show that Rboh is regulated by different factors. Among them, small GTPase are particularly interesting. In addition of their effect on RBOH regulation, they were described as negative regulators of the endocytosis (Chen et al 2012, Sorek 2010), and we know that one of the fasters osmotic stress responses is the osmotically induced endocytosis. Therefore, the ROP proteins family constitute very promising candidate of Rboh regulation in the context of the osmotic signal.

2. Objectives

In the recent years, the molecular actors that take place in the osmotic signaling cascade are starting to be elucidated. Long term and short term plant responses are already described that happen after transfer of the osmotic signal. ROS seems to have crucial role as translator between the osmotic perception and the response. They accumulate very fast as a result to osmotic stress and thus control many adaptational events, such as protein internalization, proline accumulation etc. ROS produced after osmotic stimulation is tightly regulated by non-enzymatic and enzymatic agents represented by the Rbohs. Rboh gene family is essential for many signaling pathway from development to pathogen signaling. The way, Rboh are activated by the osmotic signal remain unexplored. Hence, the main objective of this PhD thesis is to decipher the early signaling mechanism triggered after on hyperosmotic signal. This comprises the upstream regulation of the Rbohs. Ultimately, our aim is to arrive to identify new mechanism for plant osmotic sensing.

For this purpose, ROS accumulation in cells was used as a phenotypic readout of the osmotic signaling. We chose to focus on regulators of Rboh because many more molecular actors are known for this part of the ROS generating pathway. With a "bottoms up" strategy, first we used the osmotically induced ROS to find possible regulator candidates of Rboh. Considering the crucial role in the mammalian counterpart gp91, we looked at the small GTP-ase, Rho of plants (ROP). The isoform ROP6 regulate both osmotically induced ROS generating mechanisms and some of the plant's response to osmotic stimulation. We also looked at the spatial organization of ROP6 within the membrane and demonstrate that the osmotic signaling is tightly regulated by ROP6 partitioning in membrane nanodomains. Further characterization reveals that ROP6 is a central hub for plant cell signaling. This fact is questioning how specificity of information can arise. Therefore, we next wondered how ROP6 might, itself, be regulated. Because ROP6 can shuffle between active and inactive form, a characteristic common for the small-GTP-ases, we found an activator that takes part in the "on-off" shuffling called guanine nucleotide exchange factor (GEF) GEF14 which might be a ROP6 activator. More importantly GEF14 seems to be needed only for the osmotic signaling. These results will be presented and discussed in the chapter 1.

Classically, GEFs and ROPs act downstream of membrane receptors. We, therefore, continued with a gene candidate approach to investigate possible implications of some members of *Catharanthus roseus* Receptor-like kinases (CrRLK1Ls) that are known to

participate in the perception of cell wall environment which is strongly impacted during hyperosmotic stress. We first test the impact genetic or pharmacological impairment of cell wall. Then, we question the role of the cell wall sensing pathway in osmotic signaling taking the receptor kinase FERONIA as an example. Our results show that FERONIA steers GTPase signaling in plant by modulating the localization of phosphatidylserine (PS) in the membrane. PS are known to be important for GTPase residence time in nanodomains. Chapter 2 will detail aspects of the cell wall sensing and the osmotic signaling and involvement of some CrRLK1Ls over the regulation of ROP6. Finally, in the general discussion, I will propose a model for GTPase signaling with an emphasis on the role of membrane nanodomains.

3. Chapter 1: Small GTP-ase, ROP6 is an upstream signaling hub of ROS generating mechanisms

We would like to understand better the way plant perceive and transduce osmotic signal. Previous work of the lab has clearly established that osmotically induce ROS lead to cellular and physiological adaptation of plant to osmotic stress (Boursiac et al., 2008, 2005) More recently, two ROS producing machineries was identify to by induced in cell during osmotic signal (Martiniere et al., 2019). As explain earlier, the first, one is the classical PM localize NADPH oxidase that was describe in the context of many plants signaling pathways. More surprisingly, the second ROS producing machinery is based on the reduction of apoplatic iron by ascorbate, that later transfer electron to dioxygen leading to superoxide. But at this stage, how osmotic signal activated these two ROS producing machineries in cells remains totally unknown.

As explain in the introduction, RBOH activity was describe to be controlled by different type of regulators. Among them, the ROP appear particularly interesting. Beside their role as regulator of ROS, it has been shown that ROPs can regulate clathrin-mediated endocytosis. Because, we know that endocytosis regulation is tightly associate with hyperosmotic response, we though that ROP gene family might act upstream of ROS production during osmotic signaling. In the next paragraph, I will shortly introduce the ROP gene family and their role in plant signaling. Then, I will present the crucial role in osmotic signaling of a single isoform of ROP, ROP6. In particular, we have found that the way ROP is organized in the PM in response to osmotic signal is really important for cell signaling. This fact mirrors recently published works showing that ROP membrane nano-partionning is a general feature of ROP signaling. Thus, the second section will be a review article that sum up the recent advance in this field. Finally, as ROP are often implied in many different signaling pathway, the role of ROP activator protein are necessary encoding for signal specificity. In the last part, I will present some results the guanine nucleotide exchange factors (GEF) family.

3.1 INTRODUCTION

Small GTPases are often participating to signaling cascade since members of some small GTPase families are typical transducer protein acting directly downstream of receptor kinase. Mammalian small GTPase comprise of five big families (Rab, Rho, Ren, Arf and Ras) (Kahn et al., 1992). In general, Rho and Ras family members are positioned downstream of receptor kinases. Since Plants lack Ras family, Rho of plants (ROP) is the unique signaling small GTPase in plants (Figure 10). ROP proteins have been implicated in the polarized expansion of root hairs in *Arabidopsis thaliana* (ROP2 and ROP4) (Jones et al., 2002) and pollen tubes (ROP1, ROP3 and ROP5; also known as ARAC11, ARAC1 and ARAC6, respectively) (Fu et al., 2002; Jones et al., 2002; Gu et al., 2006). In the latter cell type, ROP1 has been linked to the modulation of Ca^{2+} influx at the cell tip (Li et al., 1999; Fu et al., 2001). ROP-family members have been alleged to coordinate the antagonistic interaction of actin and microtubule cytoskeletons (ROP2, ROP4 and ROP6) (Craddock et al., 2012; Lin et al., 2015) in cell shape and tissue patterning, such as in the formation of leaf epidermal pavement cells.

In addition to this, it has been shown that ROPs are not homogeneously distributed in membrane. In fact, a constitutively active form of ROP6, generated by changing Gly15 to Val in ROP6, accumulated in detergent resistant membrane (DRM), whereas in the normal case, this protein is equally distributed between DRM and the soluble part of the membrane (Sorek et al., 2007). DRM are membrane domains that are enriched with certain lipids like sphingolipids or sterols, and proteins. Those domains are associated to signaling processes. Interestingly, it has been shown that the transient S-acylation of G domain C21 and C156 in ROP6 are responsible of the diffusion and the change in the partitioning between the DRM and the soluble part (Sorek et al., 2010). More recently, Platre et al., 2019 has shown that ROP6 is able to form nanodomain in membrane minute after cell stimulation with auxin. This suggests that membrane organization of ROP protein seems to be important for their function.

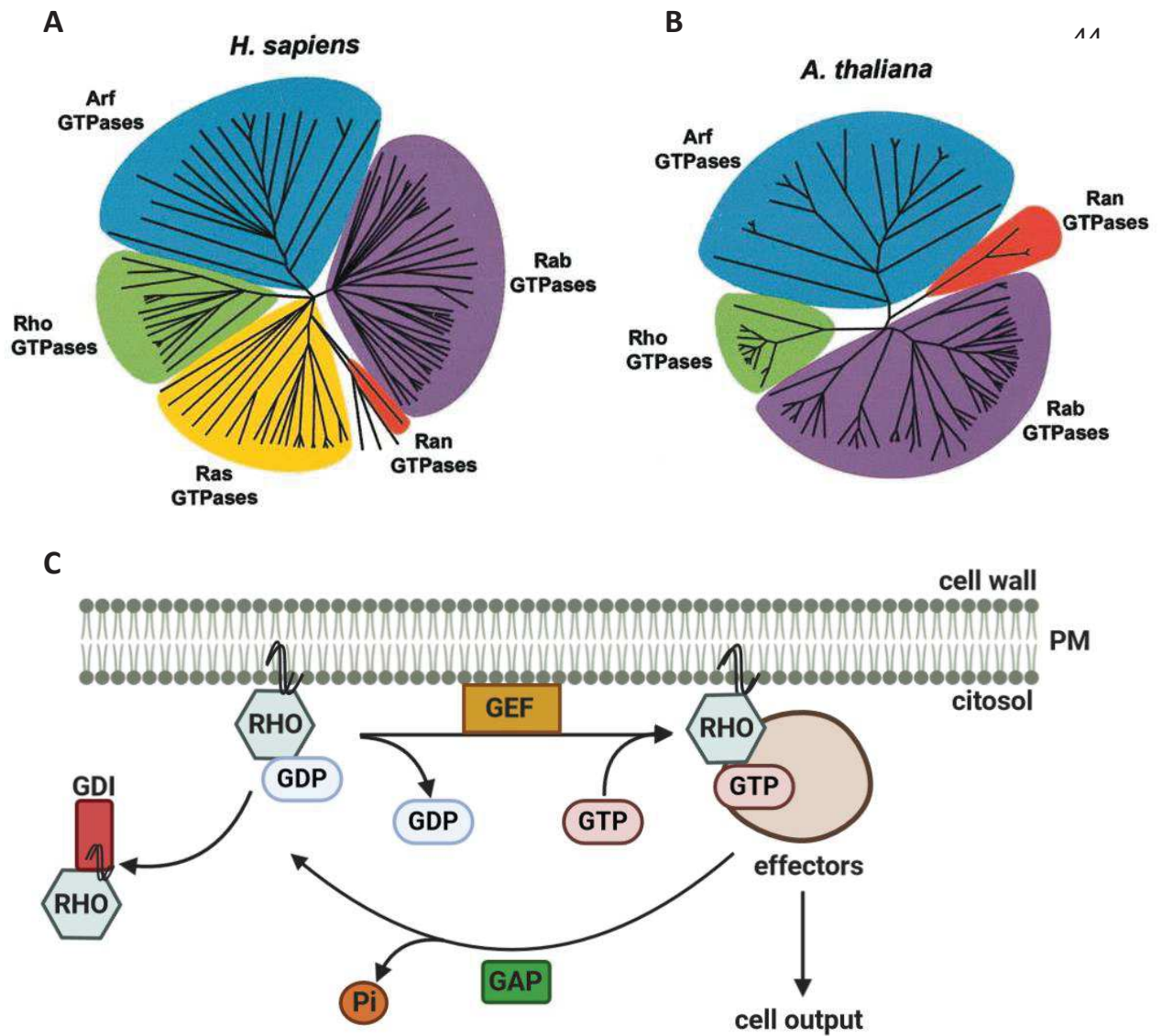


Figure 10: **Phylogenetic star diagrams of small GTPases of *H. sapiens* and *A. Thaliana* and mode of action of RHO GTPases.**

(A-B) Unrooted star diagrams were obtained using the program, ClustalW (Thompson et al., 1994). Sequences of small GTPases were obtained from the sequenced genomes of the two indicated organisms using BLAST (Altschul et al., 1997) sequence similarity searches against characterized GTPases. In each organism, distinct families (Ras, Rab, Rho, Arf, and Ran) could be distinguished, except for Arabidopsis in which no apparent Ras GTPase family could be identified. Adapted from Vernoud et al 2003. (C) Schematic representation of the GDP/GTP cycle of RHO GTPases

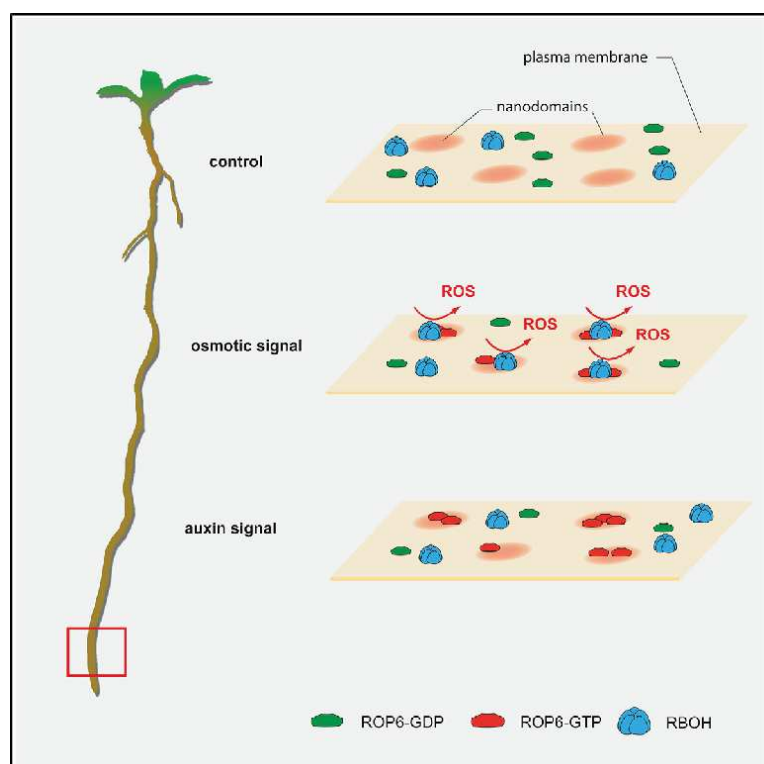
3.2 RESULTS

3.2.1 A plasma membrane nanodomain ensures signal specificity during osmotic signaling in plants

Current Biology

A Plasma Membrane Nanodomain Ensures Signal Specificity during Osmotic Signaling in Plants

Graphical Abstract



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In Brief

The mechanisms by which plants perceive and transduce osmotic signal remains incompletely understood. Here, Smokvarska et al. find that ROP6 forms nanodomain in response to osmotic signal. Then, ROP6 nanodomain recruits osmotic specific effectors that ensure downstream signal specificity

Highlights

- d An isoform of ROP GTPase mediates ROS signaling and plant responses to osmotic signaling
- d Osmotic signaling induce nanodomains that contain activated ROP6
- d Interaction of ROP6 with RBOHs enriches nanodomains locally
- d Auxin induced nanodomains containing ROP6, but not RBOHs

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Article

A Plasma Membrane Nanodomain Ensures Signal Specificity during Osmotic Signaling in Plants

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SUMMARY

In the course of their growth and development, plants have to constantly perceive and react to their environment. This is achieved in cells by the coordination of complex combinatorial signaling networks. However, how signal integration and specificity are achieved in this context is unknown. With a focus on the hyperosmotic stimulus, we use live super-resolution light imaging methods to demonstrate that a Rho GTPase, Rho-of-Plant 6 (ROP6), forms stimuli-dependent nanodomains within the plasma membrane (PM). These nanodomains are necessary and sufficient to transduce production of reactive oxygen species (ROS) that act as secondary messengers and trigger several plant adaptive responses to osmotic constraints. Furthermore, osmotic signal triggers interaction between ROP6 and two NADPH oxidases that subsequently generate ROS. ROP6 nanoclustering is also needed for cell surface auxin signaling, but short-time auxin treatment does not induce ROS accumulation. We show that auxin-induced ROP6 nanodomains, unlike osmotically driven ROP6 clusters, do not recruit the NADPH oxidase, RBOHD. Together, our results suggest that Rho GTPase nano-partitioning at the PM ensures signal specificity downstream of independent stimuli.

INTRODUCTION

Biological membranes can be seen as a patchwork where lipids and proteins are grouped in a juxtaposition of domains of various shapes and sizes. Paradoxically, membranes are also a fluid-structure allowing lateral diffusion of its constituents by thermal agitation. This property of membranes is central as it participates in the dynamic partitioning of proteins and lipids between different plasma membrane (PM) domains and consequently regulates cell-surface signaling processes [1, 2]. In plants, the vast majority of PM proteins observed with improved fluorescent microscopy technics was described to be organized in nanodomains of long dwell time (several minutes). It is especially the case of REMORIN3.1 (REM3.1), PLASMA MEMBRANE INTRINSIC PROTEIN2;1 (PIP2;1), PIN-FORMED2 (PIN2), AMMONIUM TRANSPORTER3.1 (AMT3.1), BRASSINOSTEROID INSENSITIVE1 (BRI1), RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D (RBOHD), FLAGELLIN SENSING2 (FLS2), and NITRATE TRANSPORTER1.1 (NRT1) [3–9]. Nevertheless, the functional relevance of this particular membrane organization remains poorly understood, and its role in cell signaling just begins to be explored.

Among other signals, plant cells respond to changes in water availability generated by osmotic constraints. Despite tremendous effort in the last decades, the molecular mechanisms that allow plant cells to perceive and induce early signaling events in response to osmotic stress has just begun to be understood [10, 11]. One of the first cellular responses is an accumulation of reactive oxygen species (ROS) [12] in cells, which act as secondary messengers, regulating cell endocytosis but also root water conductivity and intracellular accumulation of osmotica (e.g., proline) [13, 14]. Two processes are under action to generate ROS during osmotic signaling. One is non-enzymatic and requires reduction of apoplastic iron. The second is mediated by the PM-localized NADPH oxidases, RBOHD and F [12]. RBOHs catalyze the production of superoxide free radicals by transferring one electron to oxygen from the cytoplasmic NADPH. Even if the mechanism that drives ROS production is now better understood, it is still unclear how it is triggered by a change in osmolarity.

The Rho of plant (ROP), belonging to the super clade of Ras/Rho GTPases, have a key role in cell surface signaling including response to hormones such as auxin or ABA, but also during biotic stimulation [15]. In some cases, they also appear to

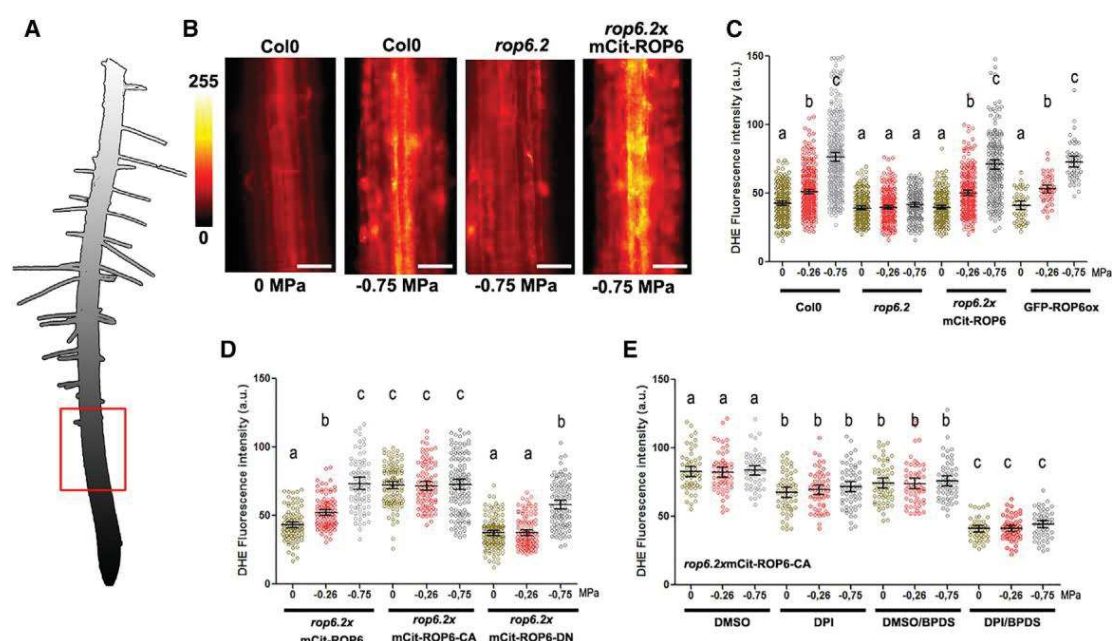


Figure 1. ROP6 Activation Is Necessary and Sufficient to Trigger Osmotically Driven ROS Accumulation in *Arabidopsis* Root Cells

(A) Drawing of *Arabidopsis* plantlets, where the red square highlights the part of root under study.

(B) Dihydroethidium (DHE)-stained root cell of Col0, *rop6.2*, and *rop6.2xmCit-ROP6* in control condition (0 MPa) or after 15 min of -0.75 MPa treatment.

(C and D) DHE fluorescence quantification after 15 min treatment with 0, -0.26 , or -0.75 MPa solution in different genetic materials: Col0, *rop6.2*, ROP6 overexpressor line (GFP-ROP6ox), and *rop6.2* lines expressing under ROP6 endogenous promoter, either ROP6 mCit-ROP6 (*rop6.2xmCit-ROP6*), the constitutive active ROP6 (*rop6.2xmCit-ROP6-CA*), or the dominant negative (*rop6.2xmCit-ROP6-DN*).

(E) ROS quantification (DHE fluorescence) in root cells expressing the constitutive active ROP6 (mCit-ROP6-CA) in control or after mild or high osmotic stimulus (respectively 0, -0.26 , and -0.75 MPa) supplemented or not with ROS enzyme inhibitors. DPI was used for inhibition of NADPH oxidase activity and BPDS to inhibit ROS produced from ferric iron.

Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p value < 0.001). $n > 49$ from 4–6 independent biological replicates. Scale bar, 20 μ m. See also Figures S1, S2 and S3.

regulate ROS accumulation, like in tip-growing cells or in response to chitin elicitation [16, 17]. ROPs are functioning as molecular switches due to a change in conformation between an active GTP-bound form and an inactive GDP-bound form. However, ROP function is also tightly associated with its lipid environment. For instance, the rice type-II ROP OsRAC1 interacts with OsRBOHB in the presence of specific sphingolipids containing 2-hydroxy fatty acids [18]. Besides, the role of charged lipids was recently exemplified in a work on a type-I ROP from *Arabidopsis thaliana*. In this study, the anionic lipid phosphatidylserine (PS) was shown to interact directly with ROP6 C-terminal hypervariable domain, to determine ROP6 organization at the PM, and to quantitatively control plant response to the phytohormone auxin [19]. Therefore, the ROP gene family may provide good candidates to regulate osmotic signaling.

Here, we show that ROP6 is a master regulator of osmotically induced ROS accumulation and participates in a set of plant responses to this signal. Using super-resolution microscopy, we found that ROP6 co-exists in the same cell in different states and that osmotic stimulation induces ROP6 nanodomain formation. These nanodomains are needed for a correct ROS accumulation in cells, and their composition differs when triggered by other stimuli, suggesting that ROP6 nanodomains may encode for signal specificity.

RESULTS

ROP6 Is Necessary for Osmotically Induced ROS Accumulation and Participates in Plant Responses to Osmotic Signal

To investigate the potential role of ROPs in osmotic signaling, we used medium or high sorbitol concentration ($c_{\text{medium}} = -0.26$ MPa and $c_{\text{high}} = -0.75$ MPa, respectively) and challenged *rop* loss-of-function mutant lines corresponding to the three isoforms that are highly expressed in roots (Figure S1A). ROS accumulation in cells, as revealed by DHE dye, was used as a fast readout for activation of osmotic signaling (Figure 1B) [12]. Compared to wild type (WT), *rop6.2* seedlings, but not *rop2.1* nor *rop4.1*, show impaired ROS accumulation (Figures 1A–1C and S1B). No additive effect was detected in *rop2.1rop6.2* or *rop2.1rop6.2xROP4RNAi* (Figure S1B). The defect in ROS accumulation observed in *rop6.2* is independent of the type of osmoticum (Figure S1C) and was fully complemented by a transgene containing mCitrine-tagged ROP6 genomic DNA driven by its promoter (*rop6.2xmCit-ROP6*, Figure 1C). The regulation of ROS signaling was extensively studied in response to PAMPs and ABA. As roots also react to stimulation with Flg22 and ABA, we tested whether some of the well-described ROS regulators are involved in osmotically induced ROS (Figures S1D and S1E). Whereas the OST1/SNRK2.6 is probably not involved in

osmotically induced ROS production, we found that knockout plants for *BIK1*, *BIK1/PLB1*, and *CPK5/6/11* show no or attenuated ROS response, respectively. This suggests a potential interaction between osmotic and PAMP signaling, as it was previously postulated [20].

Because ROS accumulation in roots has been tightly associated with deposition of the secondary wall, especially lignin [21], we wondered whether an osmotic constraint could enhance cell lignification. Roots exposed to -0.75 MPa for 24 h have a strong autofluorescence signal compared to control situation, and when stained with phloroglucinol that reveals lignin specifically, a typical cherry-red staining was observed (Figures S2A and S2B) [22]. We tested whether the osmotically enhanced lignin deposition is indeed associated with ROS accumulation. Loss-of-function plants for the two highly expressed NADPH oxidases (*RBOHD* and *F*) showed a reduced autofluorescence after exposure to -0.75 MPa, and control plants exposed to 1 mM H_2O_2 for 1 h revealed a strong fluorescent signal, showing a connection between osmotically induced lignin deposition and ROS production (Figure S2C). This response was partially regulated by ROP6, as *rop6-2* plants show dimmer root autofluorescence signal after -0.75 MPa treatments than control plants Col0 or *rop6.2xmCit-ROP6* (Figures S2C and S2D).

Interestingly, after 48 h on -0.75 MPa plate, root tip cells displayed local isotropic cellular growth (Figure S2E). This change in cell polarity has been suggested to reflect an acclimation process of the root facing hyperosmotic condition, as was described for salt or drought responses [23, 24]. Because ROPs are known to regulate cell polar growth of pavement cells, pollen tube, and root hairs [15], we wondered whether ROP6 may participate in the osmotically induced cell isotropic growth. *rop6.2* shows a significantly smaller circularity index than WT or complemented lines on treated plate (-0.75 MPa), whereas no difference between genotypes was found in control conditions (Figures S2F and S2G).

Because ROP6 seems to participate in multiple phenotypes associated to plant acclimation to osmotic constraint, we wondered if ROP6 can also participate in the changes of root growth and development. Whereas indistinguishable when 5DAG plants were transplanted in control conditions, *rop6.2* plants grew slightly faster than *rop6.2xmCit-ROP6* in -0.75 MPa plate (rate constant^{*rop6.2xmCit-ROP6*} = $0.011 \pm 0.0005 \text{ h}^{-1}$, rate constant^{*rop6.2*} = $0.009 \pm 0.0008 \text{ h}^{-1}$, t test p value = 0.02, Figures S2H–S2K). Indeed, plants have longer primary and lateral roots in loss-of-function *rop6.2* mutant in this stress condition, while no significant effect was observed for lateral root density (Figure S2L–S2N). Interestingly, ROP6 expression pattern fits a potential role in root growth, as mCit-ROP6 fluorescence is mostly present in the root meristem and elongation zone and in lateral root primordia (Figure S2O and S2P). As a whole, ROP6 appears to be necessary for osmotically induced ROS accumulation, but to some extent, it also participates in plant adaptations to hyperosmotic treatments (Figures S2D, S2G, and S2K).

ROP6 Activation, but Not Protein Quantity, Is Rate Limiting to Trigger Osmotic Signaling

Next, we tested whether ROP6 is sufficient to trigger osmotic signaling. Although GFP-ROP6ox overexpressing lines

accumulate high amounts of ROP6 proteins, no enhancement of osmotically induced ROS was observed in control condition or after osmotic treatment, suggesting that ROP activation rather than protein quantity might be a limiting factor (Figures 1C and S3A). To test this hypothesis, we used point-mutated proteins that are either constitutive active GTP-lock (ROP6-CA) or constitutive inactive GDP-lock (ROP6-DN) ROP6. Transient expression in tobacco leaves of FRET-based sensors (iROP) shows that ROP6-CA, but not ROP6-DN, interacts with the CIRB domain of PAK1, confirming their respective GTP- or GDP-lock behavior (Figures S3B–S3D). Stable *rop6.2* plants expressing mCit-ROP6-CA, under its endogenous promoter, showed a constitutively high ROS accumulation independent of the stimulus (Figure 1D). Oppositely, in *rop6.2xmCit-ROP6-DN* plants, ROS induction was attenuated after exposure to -0.75 MPa and totally suppressed after -0.26 MPa treatments, compared to a control situation (Figure 1D). This suggests that ROP6 itself might be sufficient for a part of the osmotically induced ROS production.

These results showed that ROP6 is necessary and its activation sufficient to trigger ROS production. Then, we addressed if ROP6 activation could act upstream of ROS-producing enzymes. Therefore, *rop6.2xmCit-ROP6-CA* line, which has constitutively high ROS, was treated alone or in combination with specific inhibitors for each of the two ROS pathways activated by the osmotic stimulus [12]. Diphenyleneiodonium (DPI) was used to inhibit NADPH oxidase activity and bathophenanthroline disulfonic acid (BPDS) to block ROS mediated by ferric iron [12]. In co-treatment, ROS generated by mCit-ROP6-CA is diminished drastically, suggesting that mCit-ROP6-CA is acting upstream of ROS production machinery (Figure 1E). Next, we determined if ROP6 activation is associated with a change in its subcellular localization, as described for many small GTPases [25]. A sharp fluorescent signal was observed delimiting root cells expressing mCit-ROP6, which overlaid with the FM4-64 PM dye (Figure S2Q). Only a minor difference in PM fluorescence intensity or relative PM localization was observed between WT and GTP- or GDP-lock ROP6 (Figures S3E–S3G), suggesting that ROP6 is, at most, marginally regulated by cytoplasmic/PM shuttling.

Two Populations of ROP6 Molecules Co-exist within the Plasma Membrane and Vary in Frequency Minutes after Osmotic Treatment

We recently showed that ROP6 organization at the PM is critical for auxin signaling [19]. We thus addressed whether ROP6 lateral segregation at the PM could contribute to osmotic signaling. Total internal reflection fluorescent microscopy (TIRFM) in two independent transgenic lines showed that GFP-ROP6 has a uniform localization within the PM in control conditions, while in -0.26 MPa and even more in -0.75 MPa treated cells, GFP-ROP6 appeared in diffraction-limited spots at the cell surface (Figures 2A and 2B). This suggests that ROP6 clustered in response to osmotic treatment in a dose-dependent manner (Figure 2B). Kymograph analysis showed straight lines for up to 50 s, suggesting that GFP-ROP6 clusters are stable within the PM during this period (Figure 2C). We then wondered whether ROP6 clustering could not go along with its dissociation from the PM. Indeed, GFP-ROP6 shows a lower PM association index

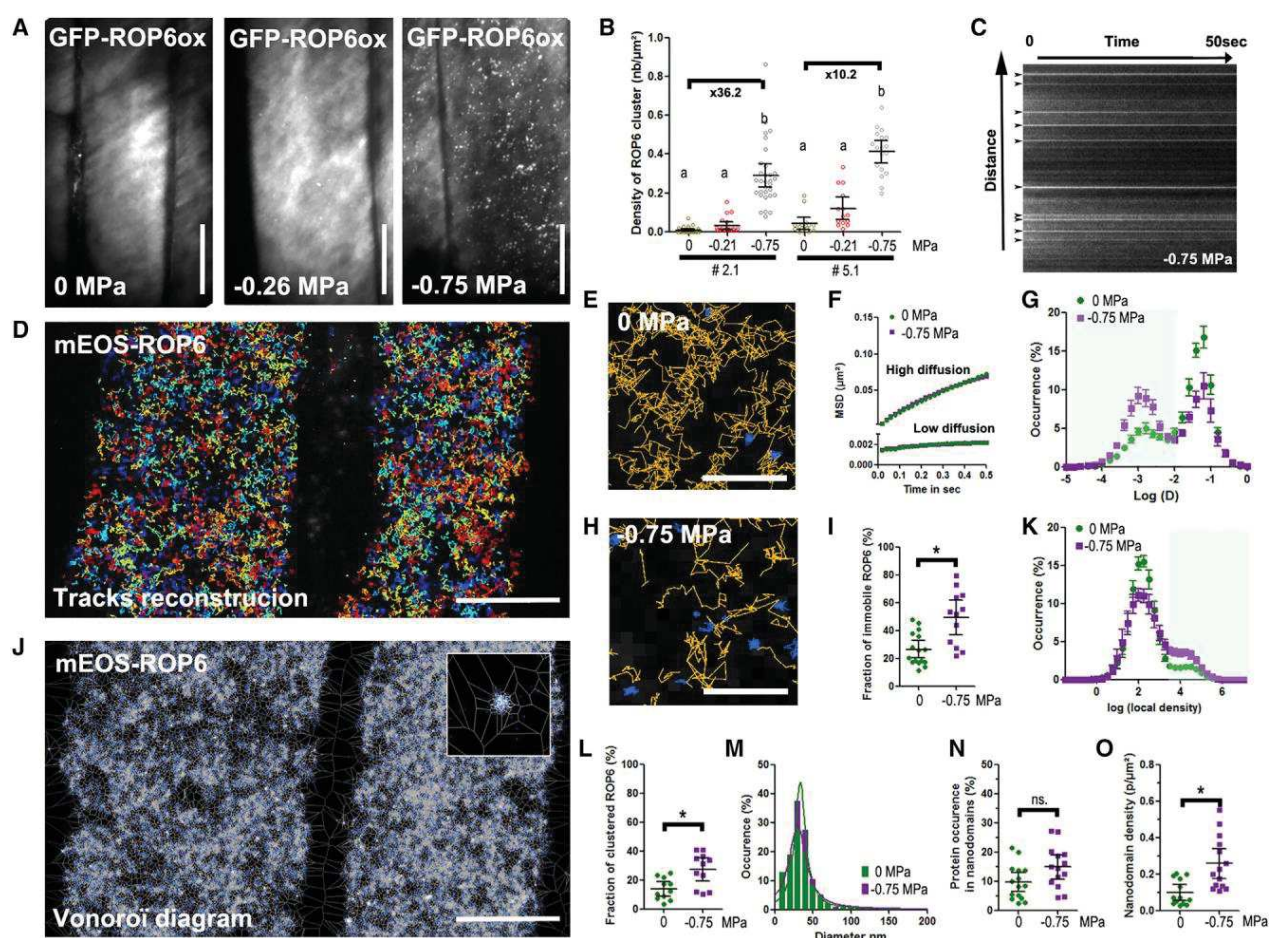


Figure 2. Osmotic Stimulus Triggers ROP6 Molecular Nanoclustering at the PM

(A) TIRFM micrograph of oxGFP-ROP6-expressing cells after 2 min incubation with solutions at either 0, -0.26 , or -0.75 MPa.

(B) Quantification of ROP6 cluster density.

(C) Kymograph image of oxGFP-ROP6 clusters from cells exposed to 0.75 MPa. Clusters at initial time point are labeled with arrows.

(D) Image reconstruction of around 5,000 single mEOS2-ROP6 molecule trajectories in two control cells.

(E) Close-up view of cell expressing mEOS2-ROP6, where trajectories with high or low instantaneous diffusion coefficient, labeled in orange or blue respectively.

(F) Mean square displacement curves of the highly or lowly diffusible molecules in control (0 MPa) or treatment (-0.75 MPa) conditions.

(G) Bimodal distribution of molecule instantaneous diffusion coefficients in control (0 MPa, green curve) and treatment (-0.75 MPa, purple curve) conditions.

(H) Close-up view of the PM of cells expressing mEOS2-ROP6 2 min after a -0.75 MPa treatment.

(I) Histogram represents the percentage of molecules with an instantaneous diffusion below $0.01 \text{ } \mu\text{m}^2 \cdot \text{s}^{-1}$ in control (0 MPa) or after treatment (-0.75 MPa).

(J) Voronoi tessellation of mEOS2-ROP6 molecules localization map from the exact same two control cells in (D). Top right inset is a close-up view showing a mEOS2-ROP6 nanodomain.

(K) Distribution of molecule local density in control (0 MPa, green curve) and treatment (-0.75 MPa, purple curve) conditions.

(L) Percentage of molecules with a $\log(\text{local density})$ higher than 3.

(M) Distribution of the mEOS2-ROP6 nanodomain diameter in control (0 MPa) and treatment (-0.75 MPa) conditions.

(N) Relative occurrence of mEOS2-ROP6 in nanodomains in control (0 MPa) and treatment (-0.75 MPa) conditions.

(O) Nanodomain density in control (0 MPa) or after 2 min treatment with -0.75 MPa solution.

Error bars correspond to a confidence interval at 95%. For (B), an ANOVA followed by Tukey test was done; letters indicate significant differences among means (p value < 0.001). * p value below 0.01 t test. $n > 12$ from 3 independent biological replicates. Scale bar, $10 \mu\text{m}$, except for (E and H), where it is $1 \mu\text{m}$. See also Figures S3 and S4 and Video S1.

after osmotic treatment (Figures S3H and S3I). When ROP6 was locked in GTP bound form (RFP-ROP6-CA), this effect was not observed although this form was able to cluster (Figures S3H–S3K). Taken together, this suggests that ROP6 clustering and its membrane dissociation are not strictly linked.

The average GFP-ROP6 spot size is close to the limit of diffraction (radius = $235 \pm 60.57 \text{ nm}$). Therefore, we next used sptPALM,

a super-resolution imaging technic recently developed on plant samples [12, 19, 26]. Upon stochastic photoswitching on live roots expressing mEOS2-ROP6, sub-diffraction spots were appearing with blinking behavior and small lifespan ($< 0.5 \text{ s}$), as expected from single-molecule behavior (Figures S4A and S4B; Video S1). By retrieving the displacement of each ROP6 single molecule along with the videos, two behaviors can be observed

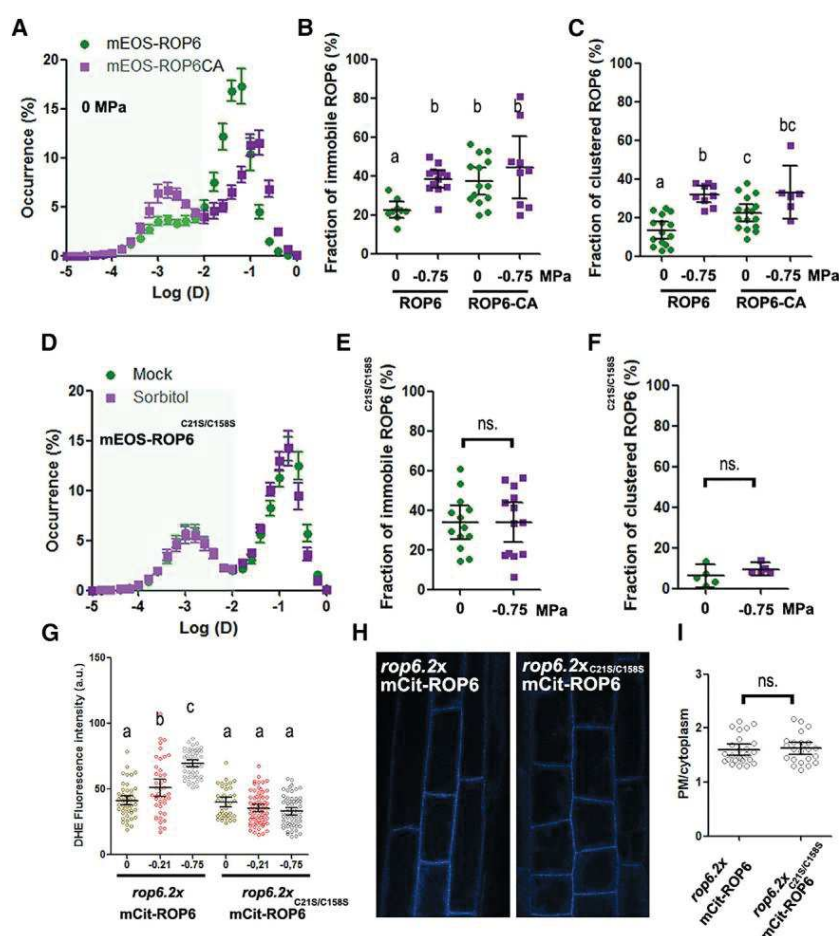


Figure 3. ROP6 Nanoclustering Is Required for ROS Accumulation

(A) Bimodal distribution of mEOS2-ROP6 instantaneous diffusion (green curve) or mEOS2-ROP6-CA (purple curve) in control condition (0 MPa).

(B) Histogram represents the percentage of molecules with an instantaneous diffusion below $0.01 \mu\text{m}^2 \cdot \text{s}^{-1}$ in mEOS2-ROP6 and mEOS2-ROP6-CA-expressing lines in control (green) or after osmotic stimulation (purple).

(C) Percentage of molecules with a $\log(\text{local density})$ higher than 3 in mEOS2-ROP6 and mEOS2-ROP6-CA-expressing lines in control (green) or after osmotic stimulation (purple).

(D) Bimodal distribution of mEOS2-ROP6^{C21S/C158S} instantaneous diffusion coefficients in control (0 MPa, green curve) and treatment (-0.75 MPa, purple curve) conditions.

(E) Histogram represents the percentage of mEOS2-ROP6^{C21S/C158S} molecules with an instantaneous diffusion below $0.01 \mu\text{m}^2 \cdot \text{s}^{-1}$ in control (0 MPa) and treatment (-0.75 MPa) conditions.

(F) Percentage of mEOS2-ROP6^{C21S/C158S} molecules with a $\log(\text{local density})$ higher than 3.

(G) Quantification of ROS accumulation by DHE staining in *rop6.2x*mCit-ROP6 or *rop6.2x*mCit-ROP6^{C21S/C158S}-expressing cells after 15 min treatment with 0, -0.26 , or -0.75 MPa solution.

(H and I) Plasma membrane localization of mCit-ROP6 and mCit-ROP6^{C21S/C158S} (H) and its relative amount at the PM (I).

Error bars correspond to a confidence interval at 95%. *p value below 0.01 t test. Ns, non-significant. $n > 9$ from 3 independent biological replicates. Scale bar, 10mm.

in control condition (Figure 2D, highly diffusible molecules in orange and lowly diffusible molecules in blue; Figure 2E). Distribution of instantaneous diffusion coefficient of ROP6 single molecules, extrapolated from mean square displacement plots, is bimodal (Figure 2F, green curve; Figure 2G). This result shows that diffusible ($D_{\text{diff}} = 0.05 \pm 0.007 \text{mm}^2 \cdot \text{s}^{-1}$) and relatively immobile ($D_{\text{imm}} = 0.002 \pm 0.0007 \text{mm}^2 \cdot \text{s}^{-1}$) mEOS2-ROP6 molecules coexist within the PM of a single cell. Minutes after -0.75 MPa treatments, the frequency of immobile mEOS2-ROP6 doubles (Figures 2G–2I). Clustering analysis on live PALM images, using Voronoi tessellation (Figure 2J) [27], showed that the occurrence of molecules with high local density increases after -0.75 MPa treatment (Figures 2K and 2L, $\text{Log}_{\text{local density}} > 3$). Nevertheless, at this stage, it was not possible to distinguish between three different cases: (1) the sizes of nanodomains are increasing after treatment; (2) cells have the same number of nanodomains between control and treatment, but with more ROP6 molecules in it; or (3) more nanodomains are formed in response to -0.75 MPa, with a similar amount of ROP6 protein. To distinguish between these possibilities, segmented images were generated based on detection local density, where only ROP6 molecules with a local density higher than $\text{Log}_{\text{local density}} > 3$ were investigated (Figures S3C–S3E). Whereas no effect on domain size nor percentage of mEOS-ROP6 molecules per nanodomains was found, the density of nanodomains per mm^2 of PM doubles after osmotic

treatment (Figures 2M–2O). Together, our results suggest that in response to osmotic stimulation, ROP6 molecules cluster in nanometer-sized domains (i.e., nanodomains), with a relatively fixed size and constant number of ROP6 molecules, and in which ROP6 barely diffuses. This ROP6 diffusion behavior differs substantially from what we know for other PM proteins, such as the P-type ATPase, AHA2, or the aquaporin, PIP1;2, which show an enhanced diffusion when cells are exposed to hyperosmotic stimulation [12].

ROP6 Nanodomains Are Necessary to Trigger Osmotically Induced ROS

Next, we addressed whether ROP6-containing nanodomains are involved in osmotic signaling. Because GTP-locked ROP6 (ROP6-CA) is constitutively producing ROS (Figure 1D), we quantified diffusion and local density of mEOS2-ROP6-CA molecules by sptPALM. In comparison to the WT protein, ROP6-CA has a higher proportion of immobile molecules and a bigger fraction of molecules with high local density in control condition. No difference was recorded between ROP6 and ROP6-CA after treatment, suggesting that ROP6-CA is constitutively associated with nanodomains (Figures 3A–3C). In addition to its C-terminal prenylation, ROP6 is transiently S-acylated on cysteines 21 and 158 [17]. These modifications are required for localization in detergent-resistant membranes and cause retarded lateral

diffusion of the constitutive active GTP-lock ROP6, but have no impact on ROP6 GTPase activity or PM targeting [17]. To test whether ROP6 acylation is required for nanoclustering, we generated mEOS2-ROP6^{C21S/C158S}-expressing plants. Using sptPALM and clustering analysis, we found that mEOS2-ROP6^{C21S/C158S} was insensitive to -0.75 MPa treatments (Figures 3D–3F). Because mEOS2-ROP6^{C21S/C158S} is not associated with nanodomains in response to osmotic treatment, we compared the ROS response in *rop6.2xmCit-ROP6^{C21S/C158S}* and *rop6.2xmCit-ROP6* complemented lines. Treatments with -0.26 MPa or -0.75 MPa did not trigger any ROS accumulation in *rop6.2xmCit-ROP6^{C21S/C158S}* (Figure 3G). Importantly, mCit-ROP6^{C21S/C158S} expressed under the control of its own promoter localized at the PM in root cells (Figures 3H and 3I), as previously reported for 35S::GFP-ROP6-CA^{C21S/C158S} in leaves [17]. Together, our results suggest that ROP6 nanodomain formation, rather than only ROP6 PM localization, is necessary to activate osmotic signaling in cells.

Activated ROP6 Interacts with RBOHD and F in PM Nanodomains to Generate ROS

We checked first whether ROP6, RBOHD, and RBOHF are co-expressed in similar *Arabidopsis* root cells. Transcriptional fusion for RBOHD and translational fusion for ROP6 and RBOHF all showed an expression signal in root epidermis (Figures S5A–S5C). Next, we tested whether the two NADPH oxidases isoforms that are activated by osmotic signal, RBOHD and RBOHF, could interact with ROP6. FLIM experiments were performed in tobacco leaf cells that transiently expressed the two putative interacting proteins tagged with GFP or mRFP. We found a significant diminution of GFP lifetime when GFP-RBOHD was co-expressed with RFP-ROP6-CA compared to cells expressing GFP-RBOHD and RFP-ROP6-DN or when cells expressed only the donor GFP-RBOHD (Figures 4A and 4B). Similar results were observed with GFP-RBOHF, suggesting that both RBOHs interact *in planta* with the GTP-, but not the GDP-locked, form of ROP6 (Figure S5D). This is in line with recent observations made in yeast in two hybrid experiments, where RBOHD and ROP6-CA were shown to interact [28].

Because ROP6 and RBOHs physically interact and ROP6 forms nanodomains that are necessary for ROS accumulation, we hypothesized that RBOHs could also be organized in nanodomains in the cell PM. *Arabidopsis* lines overexpressing GFP-tagged RBOHD and RBOHF were generated. Under TIRF illumination, GFP-RBOHD showed a uniform localization in control condition, while 2 min after -0.75 MPa treatment, cells had clearly visible spots (Figures 4C and 4D). By using GFP-RBOHDxRFP-ROP6 plants, we observed that ROP6 accumulated in the same structure as RBOHD after osmotic stimulation (Figures 4E and 4F). As *rbohF* and *rbohD* mutant plants display similarly reduced ROS accumulation in response to osmotic stimulation, we tested whether RBOHF would form stimulus-dependent clusters in the PM, as RBOHD does [12]. Even though there was a substantial number of detectable clusters in control condition, GFP-RBOHF-overexpressing plants showed an increased cluster density minutes after -0.75 MPa treatment, though less than in the case of RBOHD (Figures S5E and S5F). This last result suggests that, to some extent, both RBOHD and RBOHF have a re-localization behavior in response to

osmotic stimulation. Then, to analyze whether RBOH domain formation is a consequence of ROP6 activation or is triggered through an independent pathway, we crossed Col0 GFP-RBOHD line with RFP-ROP6-CA or *rop6.2*. The density of GFP-RBOHD clusters is much higher when the constitutively active form of ROP6 is present in cells, even in the absence of any stimulation (Figures 4G and 4H). In the case of ROP6 loss-of-function plants, GFP-RBOHD is observed in clusters in control condition, and its density did not change after cell stimulation (Figures 4G and 4I). Since GFP-RBOHD clusters are present in the absence of ROP6, and since an active ROP6 is likely required for RBOH function in response to osmoticum, these last results suggest that GFP-RBOH cluster formation is not strictly associated with ROS production.

To confirm that RBOHD/ROP6 nanodomains are acting as a functional unit for ROS production in the plant cell, we tested whether ROP6 nanodomain formation is caused and is not a consequence of ROS production. Neither inhibition of ROS by DPI/BPDS nor H₂O₂ treatment have any impact on ROP6 nanodomain formation (Figures S5G–S5I). Most importantly, we also tested whether an osmotic signal can trigger the interaction between ROP6 and RBOHD in *Arabidopsis* roots. FLIM experiment was performed in *Arabidopsis* roots expressing GFP-RBOHD and WT or constitutive active ROP6. A significant decrease of lifetime was observed 5 min after osmotic treatment with the GFP-RBOHDxRFP-ROP6 plant, but not when GFP-RBOHD was alone nor with GFP-RBOHDxRFP-ROP6-CA, which displays constitutive low FLIM as expected (Figures 4J and 4K). Thus, a RBOHD/ROP6 complex is formed in membrane nanodomains upon cell stimulation. This structure is necessary but not sufficient for osmotically induced accumulation of ROS in cells.

Can ROP6 Nanodomain Formation Mediate Independent Signaling Events?

ROP6 is necessary for several plant signaling responses, including to the phytohormone auxin [14, 18, 27]. The correct targeting of the auxin transport efflux carrier PIN2 is mediated by ROP6 and therefore participates in root gravitropic response [18, 27]. Recently, ROP6 nanodomain formation, mediated by the anionic lipid phosphatidylserine (PS), was described in response to auxin [19]. Together with our results on osmotic signaling, this suggests that nanodomain formation is a general feature of ROP6 signaling pathways in plants (Figures 5A and 5B). We addressed whether RBOHD clustering is also induced in response to auxin stimulation, as it happens after the induction of osmotic signaling pathway. No increase of GFP-RBOHD cluster density was observed in such condition, whereas ROP6 clearly shows, as expected, numerous dotted structures in the PM (Figures 5A and 5B). As it was previously described, roots exposed to auxin for a short time (60 min) failed to accumulate ROS, which contrasts with osmotic stimulation (Figure 5C) [31–34]. These results show that ROP6 nanoclusters formed after auxin or osmotic stimulations can differ in their constituent and consequently encode, to a certain extent, for signal specificity.

DISCUSSION

By combining genetic and super-resolution live imaging, we showed that ROP6 forms osmotic specific nanodomains within

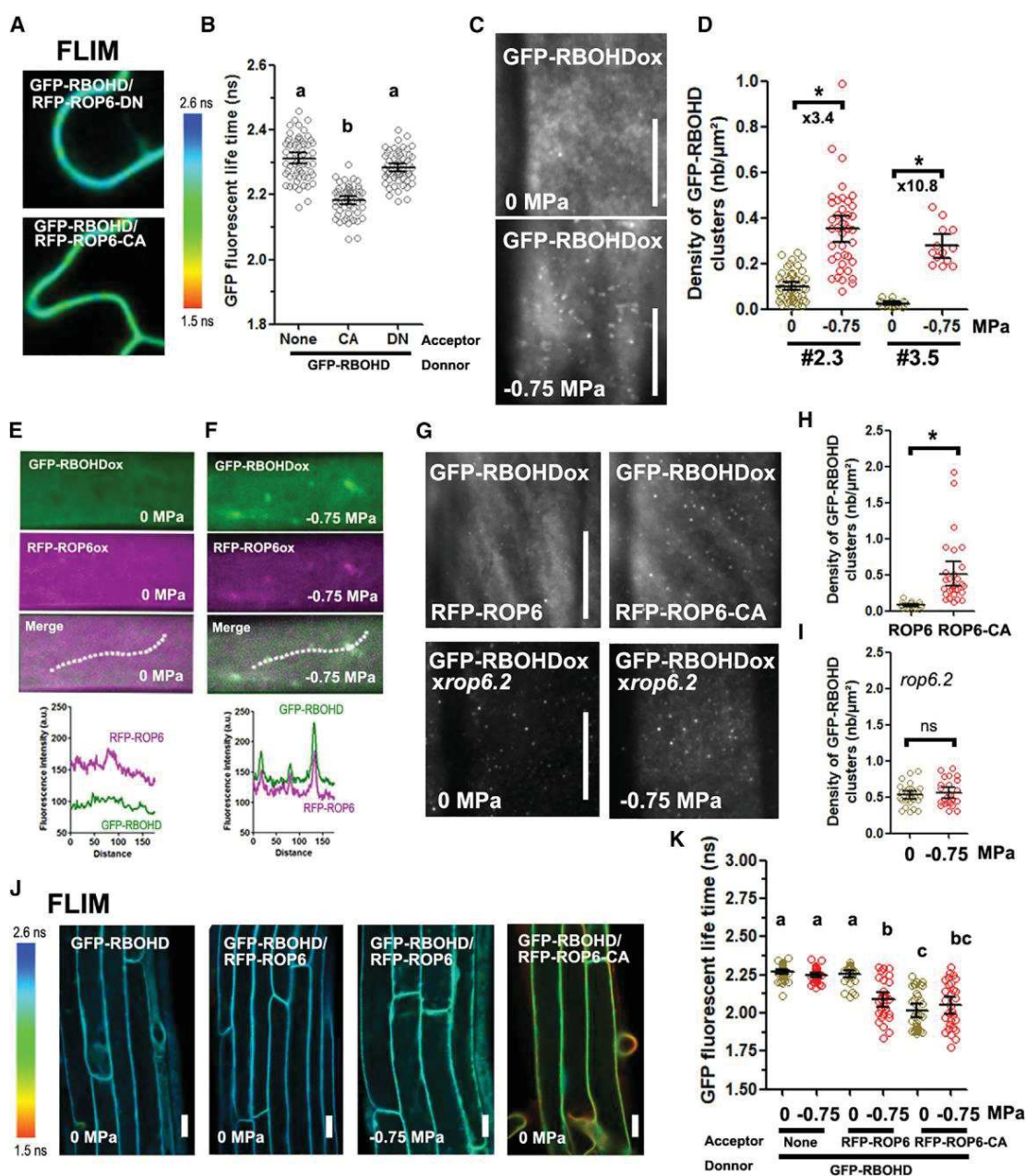


Figure 4. ROP6 Interacts and Forms Nanoclusters with RBOHD at the PM

(A and B) GFP-RBOHD fluorescence lifetime when co-expressed with dominant negative (RFP-ROP6-DN) or constitutive active ROP6 (RFP-ROP6-CA) in transient expression in tobacco leaf epidermal cells (A) and its quantification (B).

(C and D) TIRF micrograph of cell expressing GFP-RBOHD in control or after 2 min treatment with -0.75 MPa solution (C) and quantification of clusters density (D). (E and F) Cell co-expressing GFP-RBOHD with RFP-ROP6 in control (E) or after -0.75 MPa treatment (F). Graphs below represent the pixel intensity along the dotted line in each of the conditions.

(G–I) TIRFM micrograph of GFP-RBOHD signal in GFP-RBOHDxRFP-ROP6, GFP-RBOHDxRFP-ROP6-CA, and *rop6.2*xGFP-RBOHD plant in control or after -0.75 MPa incubation (G) and their respective quantification (H and I).

(J and K) GFP-RBOHD fluorescence lifetime when expressed alone or co-expressed with RFP-ROP6 or RFP-ROP6-CA in root cells (J) and its quantification (K). Error bars correspond to a confidence interval at 95%. For (B), an ANOVA followed by Tukey test was done; letters indicate significant differences among means (p value < 0.001). * p value below 0.01 T-Test. $n > 12$ from 3 independent biological replicates. Scale bar, 10 μ m. See also Figure S5.

the PM that are required to trigger secondary messenger in cells. The role of this specific ROP isoform is central for osmotic signaling, since *rop6.2* has a totally abolished osmotically

induced ROS production. In contrast, ROP2 and ROP4, which are also highly expressed in roots, are dispensable for osmotic signaling [34]. In addition, we found that ROP6 controls some

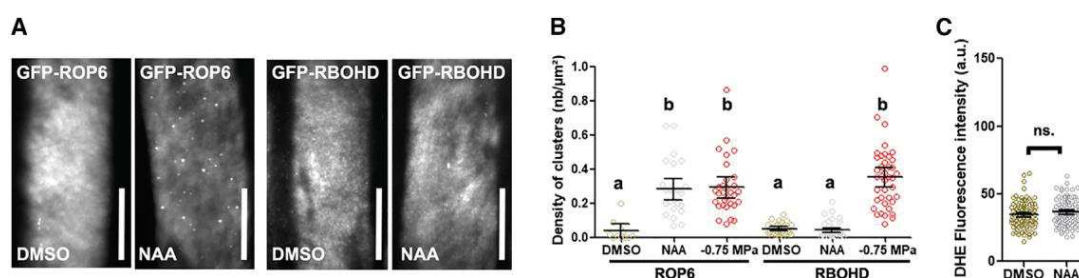


Figure 5. Auxin-Stimulated ROP6 Nanodomains Are Free of RBOHD.

(A) TIRFM micrograph of cell expressing GFP-ROP6 or GFP-RBOHD in control condition (DMSO) or after 10 mM NAA for 1 h.

(B) Cluster density quantification after NAA or -0.75 MPa treatment.

(C) Quantification of ROS accumulation by DHE staining in control (DMSO) or after 15 min treatment with 10 mM NAA. Error bars correspond to a confidence interval at 95%. For (B), an ANOVA followed by Tukey test was done; letters indicate significant differences among means (p value < 0.001). * p value below 0.01 T-Test. $n > 11$ from 3 independent biological replicates. Scale bar, 10mm.

terminal plant responses to osmotic stress. Indeed, loss of function plants for *ROP6* exhibit less osmotically induced lignin deposition in their roots. Lignin polymerization requires cellular ROS. This was, for example, demonstrated in the case of Casparian strip formation, where the NADPH oxidase, RBOHF, is localized in specific membrane domains to produce ROS that permit a spatially targeted polymerization of monolignol [21, 35]. It is therefore likely that osmotically induced lignin deposition is also mediated by ROS, but from ROP6/RBOHs nanodomains. This enhancement of lignin synthesis in response to hyperosmotic stimulation could participate in plant acclimation to stress conditions. Indeed, lignin increases cell wall stiffness and may protect cells from deformation due to turgor loss. Secondary walls are also known to counteract mineral and water leakage in roots [36, 37]. Enhanced lignin deposition may participate in such phenomena during long-term osmotic treatment. In addition, we observed that cells under elongation form spheres rather than cylinders when exposed to a hyperosmotic treatment. This tendency to isotropic cell expansion could minimize tension and consequently prevent cell bursting. ROPs are known regulators of cell polarity in pavement cells or tip-growing cells. In particular, it was demonstrated that they can participate in cytoskeleton remodeling through interaction with RIC1 and katanin [38]. Here, the need for ROP6 to ensure cell isotropic expansion and modified root elongation in response to stress condition does not necessarily involve ROS production, but could be mediated by other types of effector proteins. Thus, we believe that ROP6 is an important factor for plant osmotic signaling, likely acting right after cell osmotic perception, as ROP6 nanodomain formation happens only minutes after cell stimulation. Its role in long-term adaptation is likely more indirect.

We also demonstrated that, upon cell activation by osmotic stimulation, enhanced ROS accumulation is associated with the formation of a ROP6/RBOHD complex within the PM. Plants expressing a GTP-lock form of ROP6 show a higher cellular accumulation of ROS. In this genetic background, ROP6 nanoclustering and its colocalization with RBOHD happen without any cell stimulation. These results fit with our FLIM experiment, where RBOH interacts preferentially with ROP6 GTP-locked form. On the other hand, *rop6* plants complemented with mutated ROP6 that are unable to be acylated lose the osmotically induced nanodomain formation, and consequently, the

ROS accumulation after hyperosmotic stimulation. But how can ROP6/RBOH complex get into nanodomains? The constitutive active ROP6 (ROP6-CA) was shown to be associated with detergent-resistant membranes together with a slower diffusion [17, 19]. This behavior is mediated first by acylation of C23 and C158 residues of the protein with palmitic and/or stearic acids, and second by the direct binding between lysine residues in ROP6 hypervariable tail and phosphatidylserine (PS) [17, 19]. These results suggest, like for their animal and yeast counterpart, that small plant GTPases have a greater affinity for specific lipid environment when they are activated that then determines their nanoclustering [39–41]. Because activated ROP6 is interacting with RBOHs, we think that the former might drag and/or retain RBOH protein to ROP6 nanodomains. This is supported by the fact that ROP6-CA can alone induce RBOH clustering and that in ROP6 loss-of-function mutants, GFP-RBOHD clustering is not inducible by osmotic stimuli. Nevertheless, we also observed that the basal level of RBOH clusters in *rop6.2* is higher than in control plant. Thus, RBOH can make clusters in the absence of ROP6. In this case, however, the clusters are not associated with ROS production, suggesting that ROP6 could act as a negative regulator.

Our group has recently described that two ROS machineries are under action in response to osmotic stimulation, one of these involving two isoforms of NADPH oxidase, RBOHD and F [12]. Our results suggest that ROP6 is an upstream regulator of both ROS-generating pathways (Figures 1C and 1E). However, we also found that ROP6-DN expressed at its native level is able to partially rescue the ROS-release phenotype of *rop6.2* under high-stress conditions. Since ROP6-DN cannot interact with PAK1 nor RBOHD and F, we believe that ROP6-DN may act as a scaffold for the ROS-producing machinery that is independent of RBOH. On the other hand, the role of ROP6 on RBOH-dependent ROS production is rather associated with nanodomain formation. However, how recruitment of RBOHs in ROP6 nanodomains can regulate ROS accumulation is still unclear. Because of its ability to generate potentially harmful oxygen radicals, RBOH activity is tightly controlled in cells. This is particularly well described for pathogen elicitors, whereby several protein kinases, including BIK1 and CPK5, are necessary for PTI-mediated ROS accumulation and can directly phosphorylate RBOHD N terminus [42, 43]. The change in RBOH PM

localization, as mediated by ROP6, could participate in RBOH interaction with protein kinases and consequently alter RBOH phosphorylation/dephosphorylation kinetic. Also, RBOHD and F contain EF hands that can directly bind calcium and are essential for RBOH activity [44, 45]. Within the cell membrane, calcium gradients might exist in the vicinity of calcium membrane transporters [46]. Therefore, recruitment of RBOH proteins in ROP6-containing nanodomains that would also harbor these calcium transporters could alter RBOH micro-environment, thereby regulating its activity. In addition, RBOH dimerization was observed from purified OsRBOHB N terminus but was also suggested from step bleaching experiment done *in vivo* [7, 47]. Interestingly, we observed an epistatic interaction between *rbohD* and *rbohF* for osmotically induced ROS, suggesting that RBOHD and F might form heteromers [12]. Similar observations were recently described for ROS triggered upon cell ablation [48]. We speculate that co-clustering of RBOHD and RBOHF in ROP6-containing nanodomains could increase their probability to form functional heteromers.

Rho GTPases are generally seen as the neck of an hourglass for signal integration at the cell surface. Indeed, multiple input pathways converge on a single Rho GTPase, leading to various downstream cellular outputs that are often specific to the upstream signal. How signaling specificity is achieved in this context is an outstanding unresolved question. In our work, we found that a single ROP isoform could, in response to different stimuli, e.g., auxin and osmotic stimulus, generate very similar nanodomains in terms of shape, cellular density, or even size [19]. Nevertheless, we also found that these nanoclusters differ in their composition at least in RBOH proteins. Therefore, the segregation of signaling components in distinct plasma membrane nanodomains can generate signal specificity downstream of a single small GTPase. How this discrimination happens still remains an open question. It could be because of specific lipid environment or/and recruitment of additional proteins that will participate in the stabilization of ROP6/RBOH complexes.

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cub.2020.09.013>.

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AUTHOR CONTRIBUTIONS

M.S. and A.M. conceived the study; M.S., C.F., M.P.P., C.A., X.D., P.N., and V.B. performed experiments; M.S., A.M., J.-B.F., and P.N. analyzed data; J.-B.F. and M.N. designed the TIRF microscopes and the sptPALM analysis pipeline; C.A. gave advice about the FLIM imaging procedure; C.M., Y.J., and A.M. prepared figures and wrote the manuscript with input from all co-authors; and all authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests

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STAR+METHODS
KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti GFP, HRP conjugated	MACS	130-091-833 100ml
Chemicals, peptides, enzymes		
NaCl	Sigma Aldrich	57653-1kg
NaOH	sigma Aldrich	55881-1kg
BSA	Euromedex	04-100-812-D
TG-SDS 10x	Euromedex	EU0510-B
PBS	Euromedex	ET330
Tris	Sigma Aldrich	252859-500 g
HCL 37%	VwR-PROLABC	20252.290 1L
SDS	Euromedex	EU0660-A
Na deoxycholate	Fluka biochemica	30970 25 g
Triton-X	Sigma Aldrich	T-8532 100ml
Leupeptin	Euromedex	SP-04-2217-B10mg
PMSF	Sigma Aldrich	76307 500 g
DTT	Sigma Aldrich	43817 5g
Tween 20	Sigma Aldrich	P7949 100ml
Commasie Blue	Thermo Scientific	24592
Murashige and Skoog	Sigma Aldrich	M5519 1L
Sucrose	Sigma Aldrich	S5390 1kg
MES	Euromedex	EU0033
KOH	Sigma Aldrich	P5958 1kg
H2O2	Sigma Aldrich	216763 100ml
BPDS	Sigma Aldrich	146617 5g
NAA	Sigma Aldrich	N0640 25 g
DHE	Sigma Aldrich	07008 10mg
D-sorbitol	Sigma Aldrich	S1876 1kg
PEG 8000	Acros organics	418050010
DMSO	Sigma	D4540 100ml
Ethanol	Honeywell	603-002-00-5 1L
ABA	Sigma Aldrich	41049 250 mg
DPI	Sigma	D2926 10mg
Flg22	Proteogenix	183294
Experimental Models: Organisms/Strains:		
<i>Nicotiana tabacum</i> (SR1)	N/A	N/A
<i>Arabidopsis</i> mutant lines:		
rop6.2	NASC	SALK_091737C [29]
rop2-1	NASC	SALK_055328 [49]
rop4-1	Christine Miede	[50]
bik1	NASC	[52]
ost1-1	Nathalie Leonardt	EMS [55]
<i>Arabidopsis</i> marker lines:		
pROP6:mCit-ROP6-CA	This study	N/A
pROP6:mCit-ROP6-DN	This study	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pROP6:mCITRINE-ROP6C21S-C158S	This study	N/A
p35S:mEOS2-ROP6C21S-C158S	This study	N/A
p35S:GFP-RBOHD	This study	N/A
p35S:GFP-RBOHF	This study	N/A
p35S:GFP-ROP6	This study	N/A
p35S:RFP-ROP6	This study	N/A
p35S:RFP-ROP6-CA	This study	N/A
iROP: PAK1-mCherry-mVenus-ROP6	This study	N/A
rop6xpROP6:mCit-ROP6	[19]	N/A
p35S:EOS-ROP6	[19]	N/A
p35S:EOS-ROP6-CA	[19]	N/A
pRBOHD:nls-GUS-GFP	[20]	N/A
pRBOHF:mcherry-RBOHF	[21]	N/A
see Table S1 for further information on used <i>Arabidopsis</i> mutant alleles and marker lines.		N/A
Recombinant DNA		
See table S2 for detailed information on used plasmids.		N/A
Oligonucleotides:		
see Table S3 for detailed information on used oligonucleotides.		N/A
Softwares and algorithms:		
Fiji-ImageJ	https://fiji.sc	N/A
LAS-Software	Leica	N/A
MTT	[66, 12]	N/A
SR-Tesseler	https://www.iins.u-bordeaux.fr/team-sibarita-SR-Tesseler?lang=en , [27]	N/A
MATLAB	MatWork package	N/A
LabView	National instruments	N/A
SPCImage	Becker-Hickl	N/A
MetaMorph	Nikon	N/A
MetaFluor	Molecular devices	N/A
Ilastik	https://www.ilastik.org/	N/A

RESOURCE AVAILABILITY

Lead

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alexandre Martiniere (alexandre.martiniere@cnrs.fr)

Contact

Materials Availability

Arabidopsis lines and plasmids generated in this study are available upon request to the Lead Contact, Alexandre Martiniere (alexandre.martiniere@cnrs.fr).

Data and Code Availability

This study did not generate any code and the published article includes all dataset analyzed during this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant material

Arabidopsis thaliana accession Col-0 was used as wild type in this study. The following lines were published before: *rop6.2* [29], *rop2.1* [49], *rop4.1* [50], *rop6.2xrop2.1* [51], *rop6.2xrop2.1xROP4RNAi* [51], *rop6.2xpROP6:mCit-ROP6g* [19], *p35S:mEOS-ROP6g* [19], *p35S:mEOS-ROP6g-CA* [19], *pRBOHD:nls-GUS-GFP* [19], *pRBOHF:mcherry-RBOHFg* [21] and *bik1* [52], *bik1xpb1* [53], *cpk5/6/11* [54] and *ost1.2* [55]. Information on all genes referenced in this work, including mutant alleles and sources is provided in Table S1.

METHOD DETAILS

Growing conditions and plant materials

Plants were stratified for 2 days at 4°C and grown vertically on agar plates containing half-strength Murashige and Skoog ($3/4$ $3/4$ $3/4$ MS) medium supplemented with 1% (w/v) sucrose and 2.5mM MES-KOH pH6 for 5 days at 22°C in a 16-h light/8-h dark cycle with 70% relative humidity and a light intensity of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, prior to use. *Nicotiana tabacum* used for transient expression were grown in soil at 22°C in a 8-h light/16-h dark cycle with 70% relative humidity and a light intensity of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For root architecture analyses, seedlings were grown on vertical square 12x12 cm Petri dishes in a self-contained imaging unit equipped with a 16M pixel linear camera, a telecentric objective and collimated LED backlight. Plants were grown in the imaging automat dedicated growth chamber at 23°C in a 16-h light/8-h dark cycle with 70% relative humidity and a light intensity of 185 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Vegeled Floodlight, Colasse Seraing – Belgium). Plates were imaged every four h allowing fine kinetic analysis.

Cloning and plant transformation

The vector ROP6g/pDONRP2RP3, which includes the full ROP6 genomic sequence from ATG to the end of its 3'UTR (ROP6g – At4g35020) [19] was amplified with the overlapping primers to generate either ROP6g-CA/pDONRP2R (G15V) or ROP6g-DN/DONRP2RP3 (T20N). ROP6g-CA/pDONRP2R-P3 and ROP6g-DN/pDONRP2R-P3 were then recombined by LR multisite reaction with ROP6prom/pDONRP4P1R [19], mCITRINEnoSTOP/pDONR221 [56] and pB7m34GW [57] to generate pROP6:mCit-ROP6g-CA and pROP6:mCit-ROP6g-DN vectors, respectively. ROP6g/pDONRP2RP3 was amplified with overlapping primers to generate ROP6gC21S-C158S/pDONRP2RP3. ROP6gC21S-C158S/pDONRP2R-P3 was then recombined by LR multisite reaction with 2x35Sprom/pDONRP4P1R [58], mEOS2noSTOP/pDONR221 [19] and pB7m34GW [57] to generate p35S:mEOS2-ROP6C21S-C158S. ROP6gC21S-C158S/pDONRP2R-P3 was also recombined by LR multisite reaction with ROP6prom/pDONRP4P1R [19], mCITRINEnoSTOP/pDONR221 [56] and pB7m34GW [57] to generate ROP6prom:mCITRINE-ROP6C21S-C158S. The coding sequence of RBOHD (At5g47910), RBOHF (At1g64060), ROP6 (At4g35020), ROP6-CA (G15V) and ROP6-DN (T20N) were PCR amplified and inserted into pENTR/D-TOPO. pB7WGF2 and pB7WGR2vector were used as destination vector for respectively GFP and RFP fusion. The unimolecular FRET sensor with intact C terminus was designed based on RhoA biosensors [59]. The CRIB domain of hSPAK1 is known to interact with GTP bound form of ROP [60, 61]. We used it as a genetic probe for ROP6 GTP conformation. We ordered a synthetic gene coding for PAK1-mCherry-mVenus-ROP6 (iROP) and cloned it into pDONR221. The different binary were used either for transient expression in tobacco [62] or to generate stable *Arabidopsis* plants by floral dip method in Col-0 and then crossed with *rop6.2* line [63]. Additional information on all constructs vectors and oligonucleotides is provided in Table S2 and S3 respectively.

Osmotic and Pharmacological Treatments

Plantlets were bathed in a liquid MS/2 medium for 30 min to allow recovery from transplanting. When indicated, a pre-treatment with DPI (30min, 20mM), BPDS (50 mM, 30min), flg22 (1 mM, 30 min), ABA (1 mM, 1 h), NAA (10mM, 1 h) or H202 (1mM, 3mins for TIRF imaging and 1 h for lignin quantification) was applied. Then, plantlets were gently transferred for an additional 15 min with 5 mM of ROS dye dehydroethidium (DHE), with or without the corresponding inhibitors, into MS/2 medium (0 MPa), MS/2 medium plus 100 mM sorbitol (−0.26 MPa) for mild stress or MS/2 medium plus 300 mM sorbitol (−0.75 MPa) for severe osmotic stress. 300 g/l PEG8000 was also used to mimic severe osmotic stress. The osmotic potential of each solution was verified by point freezing osmometer (WESCOR, VAPRO 5520).

Western blot

Tissues from 5 days old Col-0, *rop6.2*mCit-ROP6g and GFP-ROP6 plantlets were grinded with liquid nitrogen to a fine powder and resuspended in 1 mL/g powder of RIPA extraction buffer (150 mM NaCl, 50mM Tris-HCl, pH- 8, 0.1% SDS, 0.5% Na deoxycholate, 1% Triton X-100, 2mM Ieupeptin, 1mM PMSF and 5mM DTT). Western blot analysis was performed with antibodies diluted in blocking solution (1% BSA in 0.1% Tween-20 and PBS) at the following dilutions: a-GFP with conjugated HRP 1:2000. Whole protein quantity was revealed with Commassie blue stain.

Sample clarification and phloroglucinol staining

Seedlings were vertically grown in half-strength MS-agar plates for 5 days and transferred on control (MS/2) or 300mM sorbitol plates for 24 h. Plantlets were treated accordingly to Malamy et al., 1997 [64]. In brief, they were incubated in 0.24 M HCl prepared in 20% ethanol, at 80°C for 15 min, and then transferred in a solution of 7% NaOH in 60% ethanol for another 15 min at room temperature. The incubated seedlings are rehydrated in subsequent baths for 5 min in 40%, 20% and 10% ethanol and infiltrated thereafter in 5% ethanol/25% glycerol for 15 min. Alternatively, root samples were stained with phloroglucinol as in Prajakta Mitra et al., 2014 [22].

ROS and autofluorescence quantification

Observations were performed on the root elongation zone using an Axiovert 200M inverted fluorescence microscope (20 \times /0.5 objective; Zeiss), with 512/25-nm excitation and 600/50 emission filters for DHE staining and with 475/28 nm excitation and 530/25 nm emission for lignin stained samples. Exposure time was 500 ms. Images were acquired using a CCD camera (Cooled SNAP HQ; PhotoMetrics), controlled by imaging software (MetaFluor; Molecular Devices). To quantify the intensity of the

fluorescence signal, the images were analyzed using ImageJ software. After subtraction of the background noise, an average mean gray value was calculated from epidermal and cortical cells.

Confocal laser scanning microscopy

Signal from *rop6.2xmCit-ROP6g*, *rop6.2xmCit-ROP6g-CA*, *rop6.2xmCit-ROP6g-DN* and *rop6.2xmCit-ROP6g^{C21S/C158S}* was imaged using Leica SP8 microscope with a 40 \times /1.1 water objective and the 488-nm line of its argon laser was used for live-cell imaging. Fluorescence emission was collected from 500–540 nm for GFP and from 600–650 nm by sequential acquisition when sample where stained 10 min with 2 mM of FM4-64. To detect FRET from the different iROP variants, images were taken with the 488-nm line of its argon laser and simultaneous detection between 515–540nm (mVenus detection) and 625–650nm (FRET channel). The ratio of FRET/Venus images was calculated with a Fiji software. Mean gray value of each cells present in the field of view was measured independently by drawing specific ROI.

TIRF microscopy

For cluster density analysis, Total Internal Reflection Fluorescence (TIRF) microscopy was done using an inverted Zeiss microscope and a 100 \times /1.45 oil immersion. Images were acquired with 50ms exposure time at 50 gain, with 475 nm excitation and 530/25 nm emission. Acquisitions were recorded for 0.5 s. Images were Z stacked by average intensity and object detection of GFP-ROP6, GFP-ROP6CA, RbohD-GFP and RbohF-GFP was made using machine learning-based segmentation with Elastik [65]. For colocalization study, TIRF microscopy was done using an inverted Nikon microscope (Eclipse) equipped with azimuthal-TIRFiLas2 system (Roper Scientific) and a 100 \times /1.49 oil immersion. One hundred images were acquired with 100ms exposure time using sequentially 488nm laser illumination with 425/20 emission filters and 561nm laser with 600/25.

FRET-FLIM

FRET-FLIM measurements were performed by multiphoton confocal microscopy (ZEISS LSM 780) with the method of measuring the lifetime of photons (TCSPC: Time correlated single photon counting) and under a 40 \times /1.3 oil immersion objective (Peter and Ameer-Beg, 2004). The GFP (donor GFP-RBOHD or GFP-RBOHF) was excited with 920 nm by a pulsating infra-red laser Ti:Saphir (Chameleon ULTRA II, COHERENT) during 90 s and the emitted fluorescence was collected by HPM-100 Hybrid detector. The decreasing fluorescence curve was obtained with the SPCImage (Becker-Hickl) software for each zone of interest. The lifetime of the GFP was estimated based on a regression curve, either mono-exponential when the donor was expressed alone and bi-exponential when the donor was expressed in the presence of the acceptor proteins (RFP-ROP6, RFP-ROP6-CA and RFP-ROP6-DN). Three biological repetitions were done and for every biological replicate, 5 cells were analyzed.

sptPALM

Root cells were observed with a homemade total internal reflection fluorescence microscope equipped with an electron-multiplying charge-coupled device camera (Andor iXON XU_897) and a 100 \times /1.45 oil immersion objective. The coverslips (Marienfeld 1.5H) were washed sequentially with 100% ethanol, acetone and water. Then, they were bathed with a 1M KOH solution and then ultrasonicated for 30 min. After several wash-outs with MilliQ water, they were dried under Bunsen burner flame. The laser angle was adjusted so that the generation of the evanescent waves give a maximum signal-to-noise ratio. The activation of the photoconvertible tagged mEOS-ROP6, mEOS-ROP6-CA and mEOS-ROP6^{C21A/C158S} was done by a low-intensity illumination at 405 nm (OBIS LX 50mW; Coherent), and 561 nm (SAPPHIRE 100mW; Coherent) emission combined with a 600/50 (Chroma) emission filter was used for image acquisition [12]. Ten-thousand images were recorded per region of interest and streamed into a LabVIEW software (National Instruments) at 20ms exposure time. Ten to 20 cells/treatment were analyzed out of three biological replicates.

Single-Particle Tracking and Voronoi Tessellation

Individual single molecules were localized and tracked using the MTT software [66]. Dynamic properties of single emitters in root cells were then inferred from the tracks using a homemade analysis software written in MATLAB (The MathWorks) [12]. From each track, the MSD was computed. To reduce the statistical noise while keeping a sufficiently high number of trajectories per cell, tracks of at least five steps (i.e., ≥ 6 localizations) were used. Missing frames due to mEOS blinking were allowed up to a maximum of three consecutive frames. The diffusion

coefficient D was then calculated by fitting the MSD curve using the first four points. For the clustering analysis, the positions returned by MTT of each mEOS detection were used as input to the SR-Tesseler software [27]. Correction for multiple detection was made based on recommendation from Levet et al., 2015 [27]. The local densities of each track were calculated as the invert of their minimal surface. Then, nanocluster size, relative number of ROP6 molecules in nanodomains and density of nanoclusters were calculated after defining region of interest (ROI) where the local density was 50 times higher than the average. Only ROI with at least 25 detections were considered.

Statistical Analysis

For each condition or treatment, 9–12 cells were analyzed from at least 5–7 different seedlings. All experiments were independently repeated 2–3 times. Data are expressed as mean \pm 95% confidence interval. ANOVA followed by Tukey test was done, letters indicate significant differences among means (pvalue < 0.001). * p value below 0.01 Student T-Test. Statistical analyses were performed in GraphPad Prism (GraphPad Software).

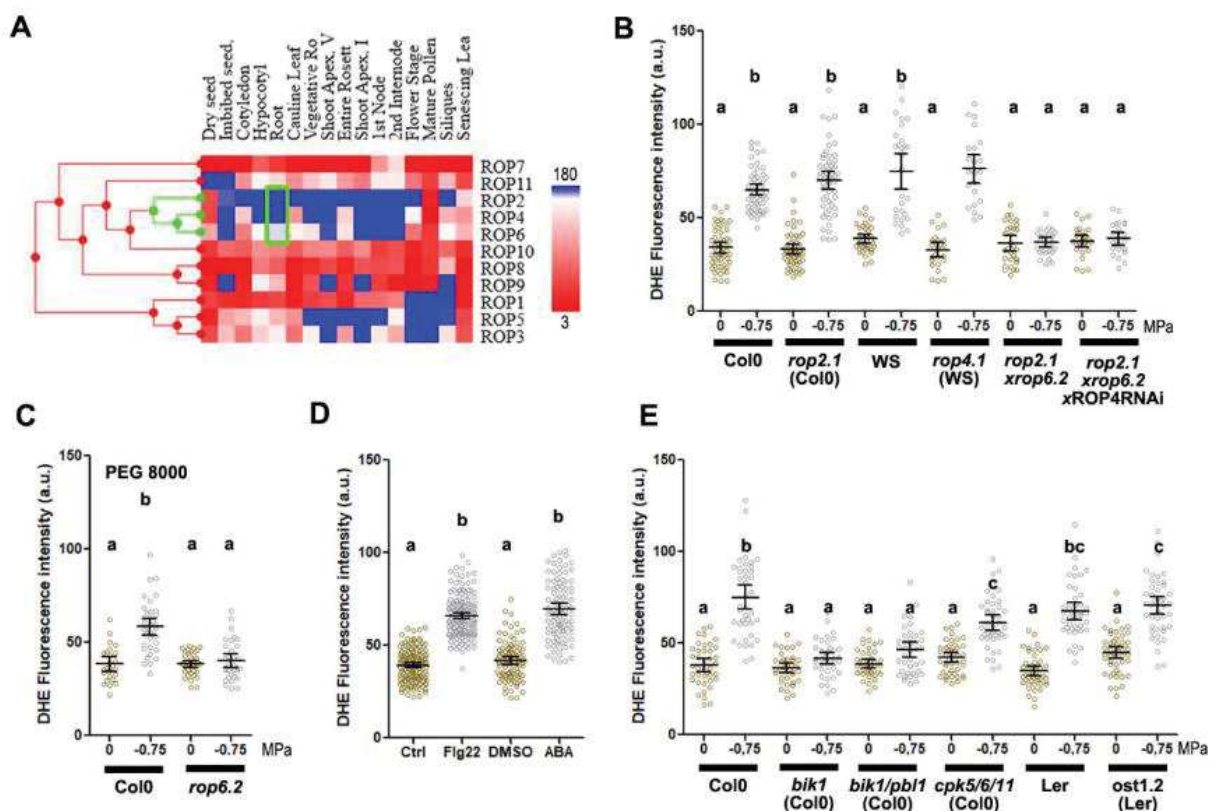
Current Biology 30, 1–11.e1–e4,
December 7, 2020 e4

Current Biology, Volume 30

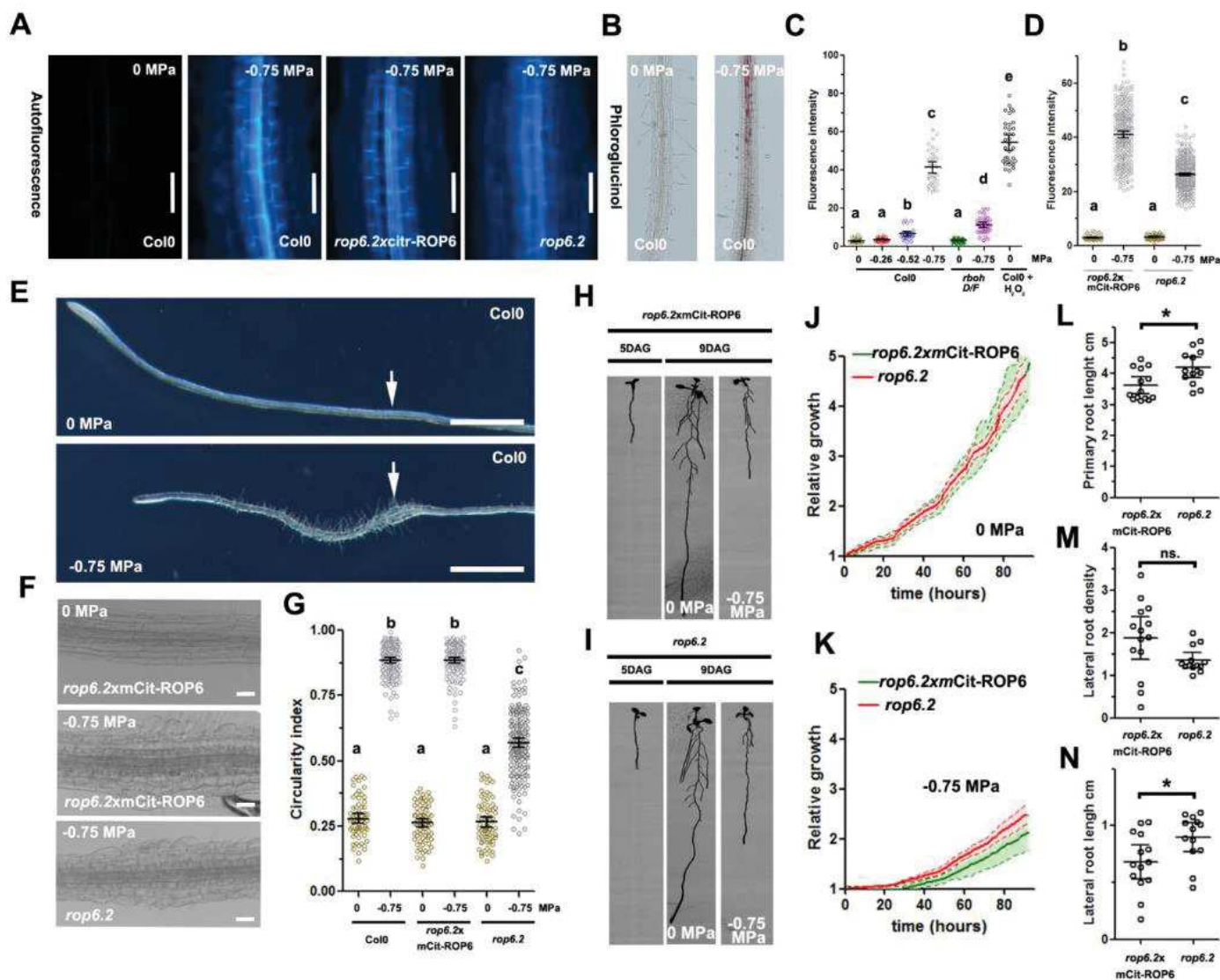
Supplemental Information

A Plasma Membrane Nanodomain Ensures Signal Specificity during Osmotic Signaling in Plants

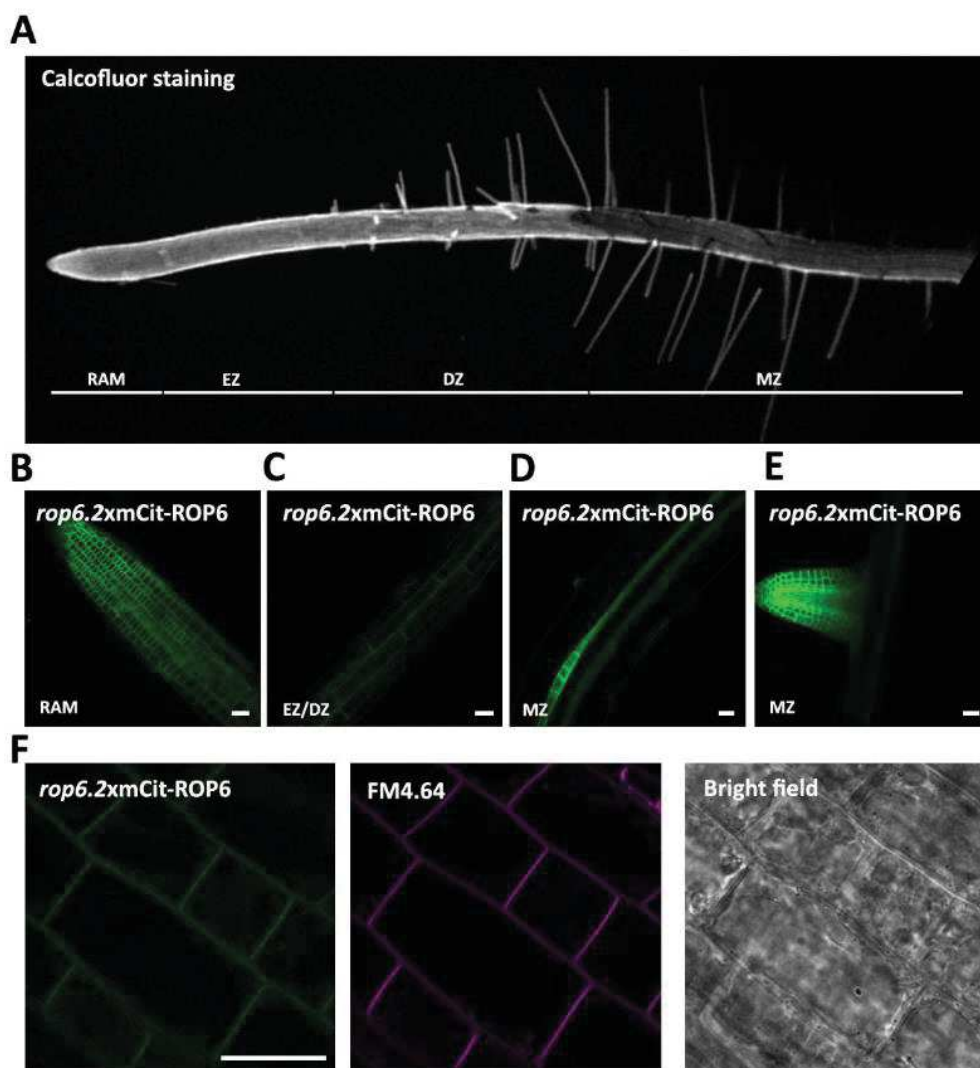
Marija Smokvarska, Charbel Francis, Matthieu Pierre Platre, Jean-Bernard Fiche, Carine Alcon, Xavier Dumont, Philippe Nacry, Vincent Bayle, Marcelo Nollmann, Christophe Maurel, Y. Jaillais, and Alexandre Martiniere



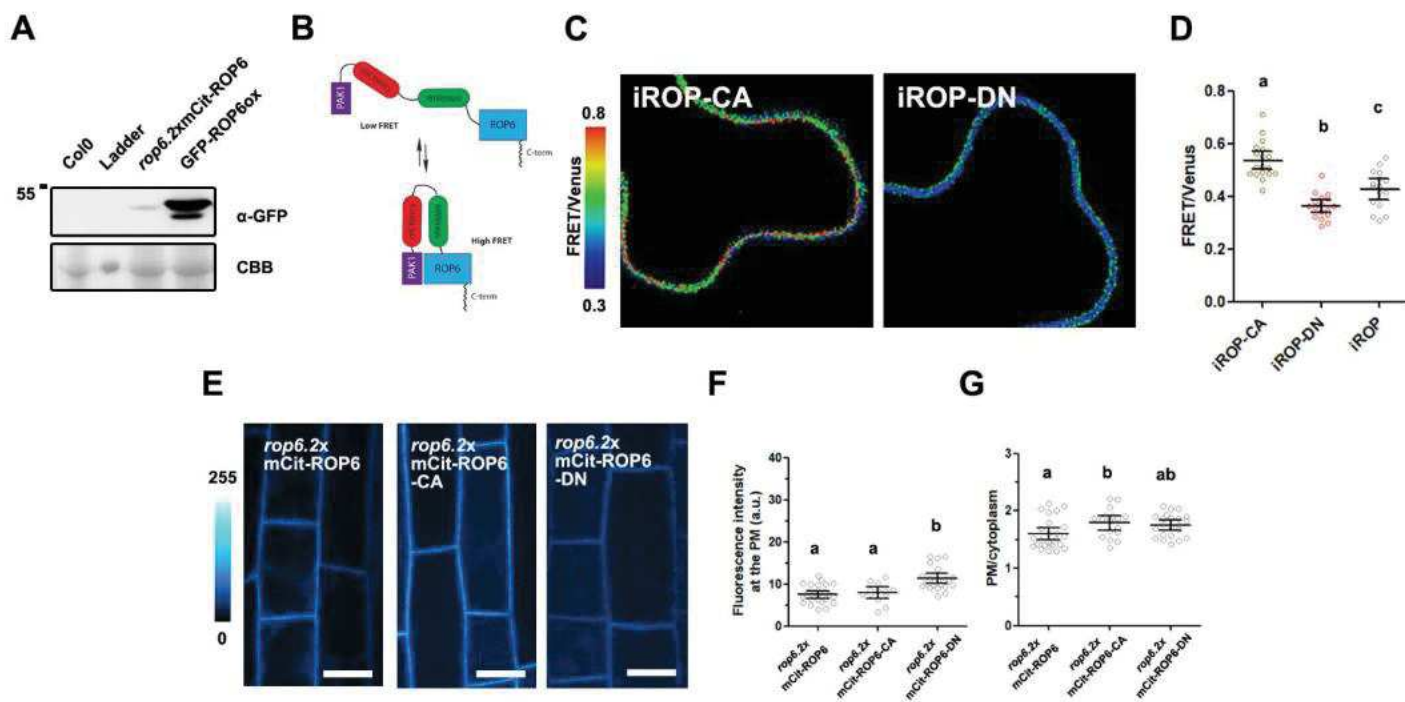
FigS1: Expression pattern of different ROP isoforms and the ROS production phenotype of single and multiple mutants. (A) Gene expression clustering of the different ROP isoforms based on eFP-browser databases. Green square shows the three isoform highly express in root tissue (ROP2, ROP4 and ROP6). (B-E) Quantification of ROS accumulation (DHE staining) in control or after 15 minutes of -0.75 MPa treatment in the indicated genotype, with either sorbitol (B and E), PEG8000 (C), flg22 (D) or ABA (D). Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test was done, letters indicate significant differences among means (p-value < 0.001). n > 21 from at least 2 independent biological replica.



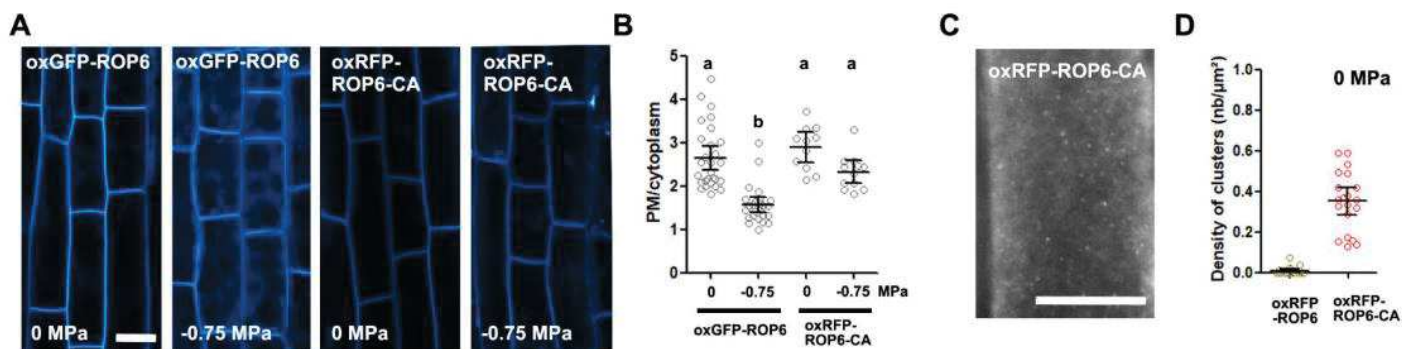
FigS2: ROP6 participates to lignin accumulation, cell isotropic growth and root elongation in response to osmotic stimulus. (A) Cell autofluorescence of *rop6.2* and complemented lines expressing mCit-ROP6 under ROP6 endogenous promoter in control plate or after -0.75 MPa treatment for 24 hours. (B) Phloroglucinol staining, that show pink precipitate when in complex with lignin in control condition or after -0.75 MPa treatment for 24 hours. (C) Cell autofluorescence quantification of Col(0) plant exposed for 24 hours to control, -0.26, -0.5, -0.75 MPa. As comparison, cell autofluorescence was also observed in *rbahDxrbohF* line was exposed to -0.75 MPa and Col(0) treated for 1 hour with 1mM H₂O₂ treatment. (D) Cell autofluorescence quantification in *rop6.2* and *rop6.2xmCit-ROP6* in control or treated plate (-0.75 MPa). (E) 2 days after transfer on -0.75 MPa plate, root cells present inflated cells (arrow). The arrows are located at the point where the root tip was at the time of transfer. (F) Close up view of cells in this zone in control condition or after treatment (-0.75 MPa) for *rop6.2xmCit-ROP6* or *rop6.2*. (G) Quantification of cell circularity index. (H-N) the complemented line (*rop6.2xmCit-ROP6*) or the mutant *rop6.2* were grown 5 days on control plates and then transferred for 4 more days in either control condition or on plate supplemented with osmoticum to reach -0.75 MPa of water potential. Relative growth of *rop6.2xmCit-ROP6* or *rop6.2* in control (J) or in -0.75 MPa plate (K). Quantification of the primary root length (L), lateral density (M) and lateral root length (N) of *rop6.2xmCit-ROP6* or *rop6.2* grown on -0.75 MPa plate. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test was done, letters indicate significant differences among means (pvalue<0.001). N=3 independent replica. Scale bar 20 μ m for (A) and 2 mm for (E). n>14 from 3 independent biological replica.



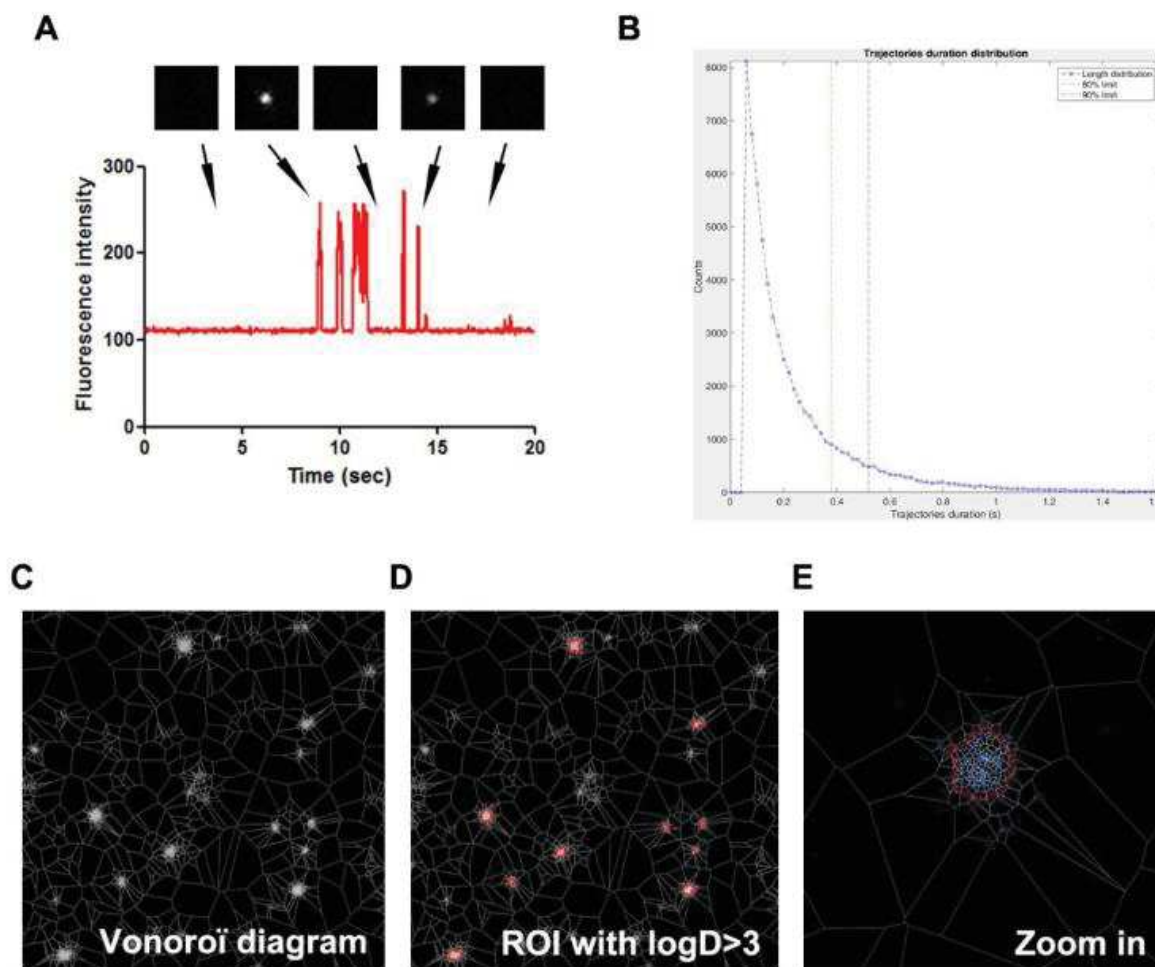
FigS3: **Expression pattern of *rop6.2xmCit-ROP6* lines along the root.** (A) Arabidopsis control plant counterstain with calcofluor bright to illustrate the different root zone. Root apical meristem (RAM), elongation zone (EZ), differentiation zone (DZ) and mature zone (MS). (B-E) Representative micrograph of the fluorescent signal observed in *rop6.2* lines complemented with mCit-ROP6 under ROP6 endogenous promoter. (F) mCit-ROP6 signal is mostly visible at the cell PM, reveal by FM4-64 staining. Scale bar 20 μ m.



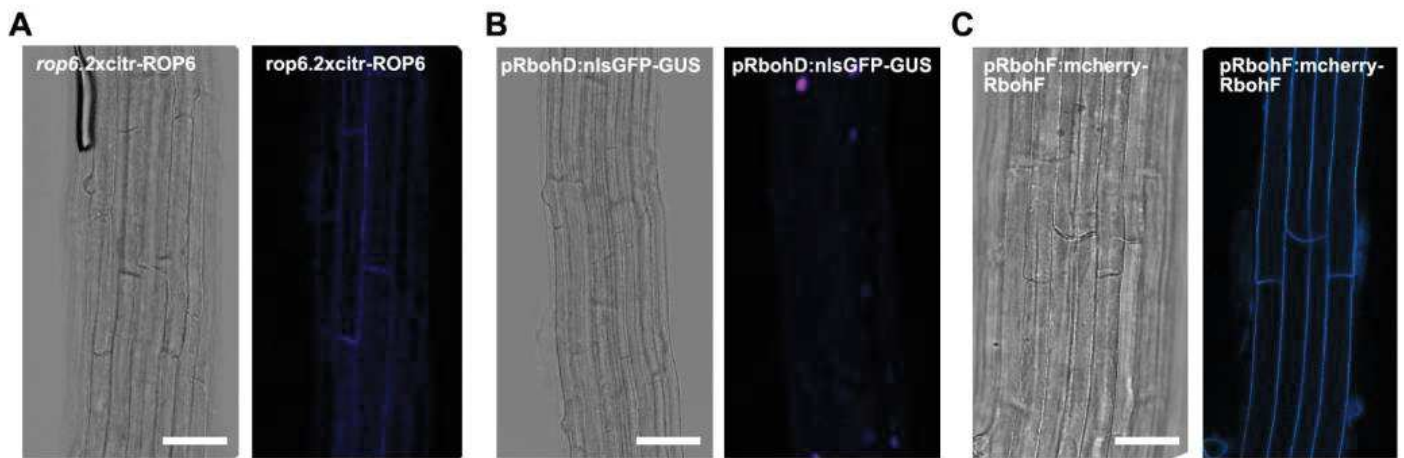
FigS4: Characterization of GFP-ROP6 overexpressing line, detection of ROP6 activation with iROP sensor and localization of *rop6.2xmCit-ROP6*, *rop6.2xmCit-ROP6-CA* and *rop6.2xmCit-ROP6-DN*. (A) Western blot with antibody against GFP on plant protein extract from Col(0), ROP6 complemented line (*rop6.2xmCit-ROP6*) and ROP6 overexpressing line (ROP6). (B) Schematic of iROP sensor. GTP-bound form ROP6 interact with PAK1, allowing FRET between Venus and mCherry. At this opposite, if ROP6 is inactive in its GDP-bound form the distance between the two fluorescent proteins increase diminishing FRET efficiency. (C) Ratio images of transient expression of iROP sensors lock in GTP form (iROP-CA) or in GDP form (iROP-DN). (D) Relative variation of FRET between iROP-CA, iROP-DN and iROP. (E) Confocal micrograph showing the localization of wild type ROP6 (*mCit-ROP6*), ROP6 constitutive active ROP6 (*mCit-ROP6-CA*) and the dominant negative ROP6 (*mCit-ROP6-DN*) and its respective fluorescence and its relative amount at the PM (F and G). CBB, Coomassie brilliant blue. $n > 14$ from at least 2 independent biological replica



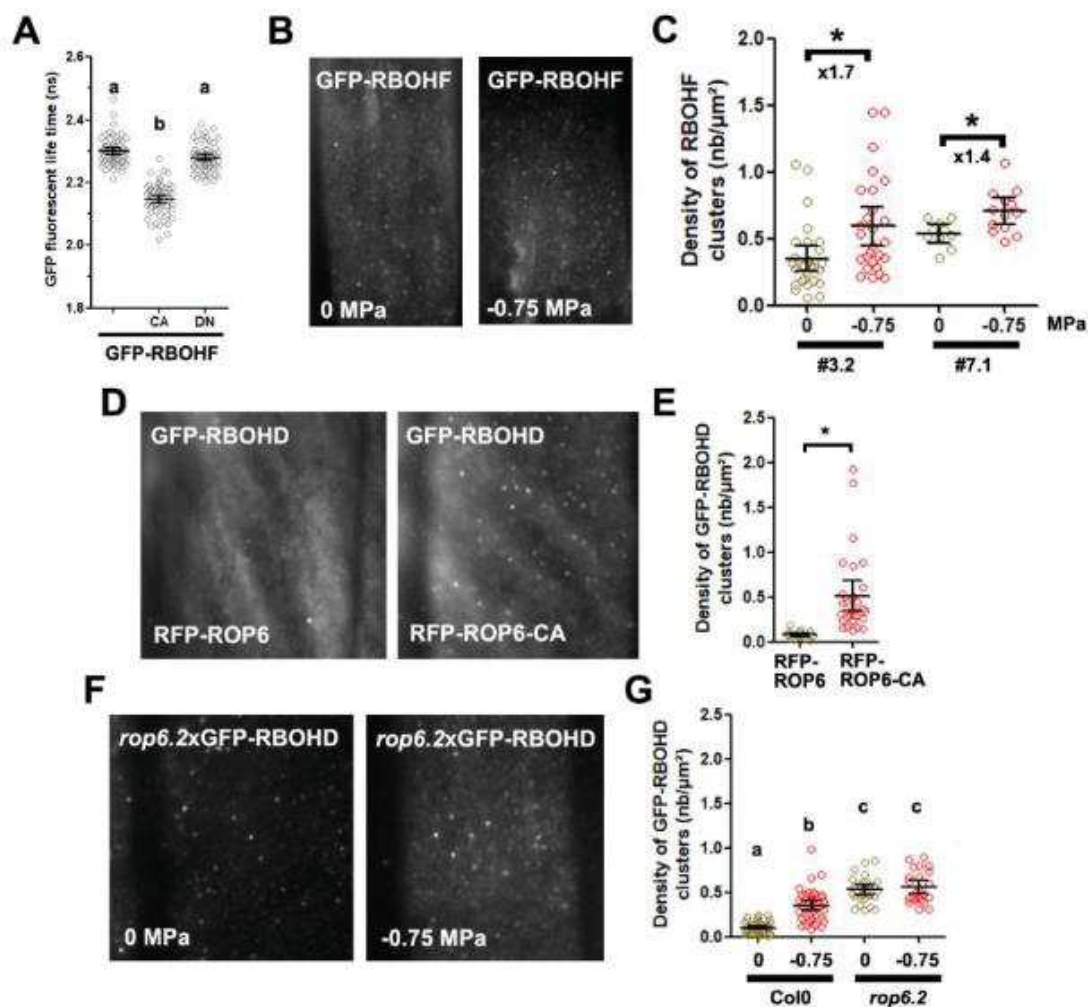
FigS5: Cellular localisation of oxGFP-ROP6. (A) Confocal micrograph showing the localization of oxGFP-ROP6 and oxRFP-ROP6-CA in control condition and after -0.75 MPa treatment and its respective quantification (B). (C) TIRFM micrograph of oxGFP-ROP6-CA expressing cells. Quantification of clusters density of oxGFP-ROP6 and oxRFP-ROP6-CA in control condition. $n > 11$ from 2 independent biological replica



FigS6: ROP6 single-molecule imaging and Voronoi tessellation. (A) To verify that we are indeed recording single mEOS2-ROP6 molecules, we plot fluorescence intensity of a typical mEOS2-ROP6 sub-diffractive cluster along time. The signal intensity observe is not continuous and the OFF state vary in duration between seconds and milliseconds. This blinking behaviour is typical from single-molecule observation. We also quantify the track duration (B). As expected from single molecules, vast majority of the tracks do not last for more than 0.5 seconds. (C) Picture of Voronoi diagram, where each point/seeds correspond to a mEOS2-ROP6 localization and edges of Voronoi cells are represented in white. (D) Segmented region of interest (ROI) with a particle local density greater than $\log(\text{local density}) > 3$ (ROI appear in red). (E) -Close up view of one ROP6 nanodomain where each blue dots represent one mEOS2-ROP6 localization.



FigS7: **ROP6, RBOHD and RBOHF are expressed in root epidermal cells.** Expression pattern of the translational fusion pROP6:mCit-ROP6 (A) and pRBOHF:mCherry-RBOHF (B) and the transcriptional fusion pRBOHD:nls-GFP-GUS (C).



FigS8: RBOHD and RBOHF interaction with ROP6 and localization in response to osmotic stimulus. (A) Quantification of GFP-RBOHF fluorescence life time expressed in transient expression in tobacco leaf epidermal cells, either alone, or co-expressed with the dominant negative (RFP-ROP6-DN) or the constitutive active ROP6 (RFP-ROP6-DN). (B) TIRFM micrograph of cell expressing GFP-RBOHF in control or after 2 minutes treatment with -0.75 MPa solution and quantification of clusters density (C). (D and F) TIRFM micrograph of GFP-RBOHD signal in GFP-RBOHDxRFP-ROP6, GFP-RBOHDxRFP-ROP6-CA and *rop6.2*xGFP-RBOHD plant in control or after -0.75 MPa incubation and their respective quantification (F and G). Error bars correspond to a confidence interval at 95%. For (A), ANOVA followed by Tukey test was done, letters indicate significant differences among means (p -value <0.001). * p -value below 0.01 T-Test. $n>9$ from 3 independent biological replica. Scale bar 10 μm .

3.2.2 Function of membrane domains in Rho-Of-Plant signaling

Meanwhile this work was made some papers come out that emphasize the role of nanopartitioning in Rho of plant signaling (Pan et al., 2019; Platre et al., 2019). Especially, it was shown that ROP6 nanodomains can mediate auxin signaling in plant. In addition, its interaction with phosphatidylserine (PS), a minor anionic lipid of the PM, can regulate GTPase residence time in nanodomains and modulate downstream auxin plant response (Platre et al 2019). These results, with ours, emphasize the role of membrane in ROP signaling. The following manuscript review recent advances in this field and propose new views on the impact of membrane nanodomain on ROP signaling.

3.2.2 Function of membrane domains in Rho-Of-Plant signaling

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Abstract

In a crowded environment, the time needed to establish interactions between different molecular partners can take a long time. Biological membranes have solved this issue, being at the same time fluid; as their components diffuse within the 2D of the membrane, but also compartmentalized in domains. This nanoscale organization of the membrane creates emergent properties that determine cellular signaling. Rho of Plant (ROP) are small GTPases involved in hormonal but also biotic and abiotic signaling as well as fundamental cell biological properties such as polarity, vesicular trafficking or cytoskeleton dynamics. Their association to the membrane is essential for their function, as well as their precise targeting within micrometer-sized polar domains (i.e. microdomains) and nanometer-sized clusters (i.e. nanodomains). Here, we review our current knowledge about the formation and the maintenance of the ROP domains in membranes. Furthermore, we propose an hypothetical model for ROP membrane targeting and discuss how the organization of the ROP in

membrane could determine signaling parameters like signal specificity, amplification and integration.

Advances: 4-5 bullet points

- ROPs regulate cell polarity and are themselves polarly localized, often via self-organizing systems involving GEF and GAP proteins
- The organization and dynamics of ROP membrane localization at the nanoscale is pivotal for their function
- Lipids modifications and lipid interactions are involved in ROP polarity, nanodomain formation and function
- Anionic lipids acts as signaling rheostat that modulates signaling output during development
- The input-specific composition of ROP nanodomains contribute to output specificity

Outstanding questions: 4-5 bullet points

- How, where and when are ROPs getting S-acylated and de-S-acylated?
- What are the similarities and differences between the mechanisms that establish the localization of ROPs in polar domains and nanodomains? and can we extrapolate concepts established on polarity to the nanoscale?
- How does ROP nanoclustering, not just GTP-loading, control effector activation? and to what extent does it contribute to regulate signaling in a quantitative manner?
- How are ROPs getting immobilized at the plasma membrane once activated? and what is the impact of the cell wall, lipids and membrane receptors in this process?
- Can nanodomains containing different ROPs and/or different effectors co-exist within the same cell? and if yes, what are their impacts in signaling integration and specificity?

Introduction

Rho of Plant (ROP) are members of the small GTPase family of about 20 kDa, essentially made of their GTPase domain (also called G domain) with short N- and C-terminal extensions (Figure 1A). They are defined by their basic biochemical activity of binding guanosine triphosphate (GTP) and hydrolyzing it into guanosine diphosphate (GDP), also known as GTP/GDP cycle (Figure 1C) (Bourne et al., 1991). Classically, the GDP-bound form is inactive, while the GTP-bound form is active and can associate and activate downstream proteins (hereafter referred to as “effectors”) (Figure 1B). This process represents a ubiquitous regulatory mechanism in cells, making small GTPase molecules behaving like a digital switch (Henis et al., 2009). The control of these switches comes via the association of GTPases with additional proteins. First, guanine nucleotide exchange factors (GEFs) catalyze the conversion of small GTPase from their GDP-bound state into their GTP-bound state, therefore, placing them in their “active” conformation (Figure 1B). GEFs also facilitate the release of the small GTPase activating proteins (GAPs) that increase GTP hydrolysis and serve as small GTPase inhibitors (Figure 1B) (Berken and Wittinghofer, 2008). A third regulatory element of some small GTPases is the guanosine nucleotide dissociation inhibitors (GDIs) which prevent activation by keeping GDP-bound GTPases from localizing to membranes (Figure 1B) (Cherfils and Zeghouf, 2013).

Small GTPase is one of the largest families of signaling molecules in all eukaryotic cells. It is divided into five distinct families: Ras, Rab, Arf, Ran, and Rho (Kahn et al., 1992). Members of the Rab and Arf families mostly function in the regulation of vesicular trafficking, including the formation of vesicles and tubules, regulation of organelle dynamics via interaction with cytoskeleton components, vesicle docking on acceptor membranes, the specification of membrane identity or compartment maturation (Gillingham and Munro, 2007). Ras-related nuclear proteins (Ran) on the other hand regulate the transport of proteins and RNA across the nuclear envelope (Kalab et al., 2002). Aside from nucleocytoplasmic transport, Ran has also been implicated in a variety of other cellular functions such as proper mitotic spindle assembly for chromosome alignment during mitosis (Clarke and Zhang, 2008); and increased apoptosis and suppression of cell proliferation in human cells (Deng et al., 2013). The two last families of eukaryotic small GTPases are often grouped in the so-called Ras superfamily and are involved in cell surface signaling. Members of the Ras subfamily are well-described regulators of cell proliferation downstream of growth factor signaling, while members of the Rho subfamily are mainly involved in the regulation of cytoskeleton dynamics, especially actin filaments.

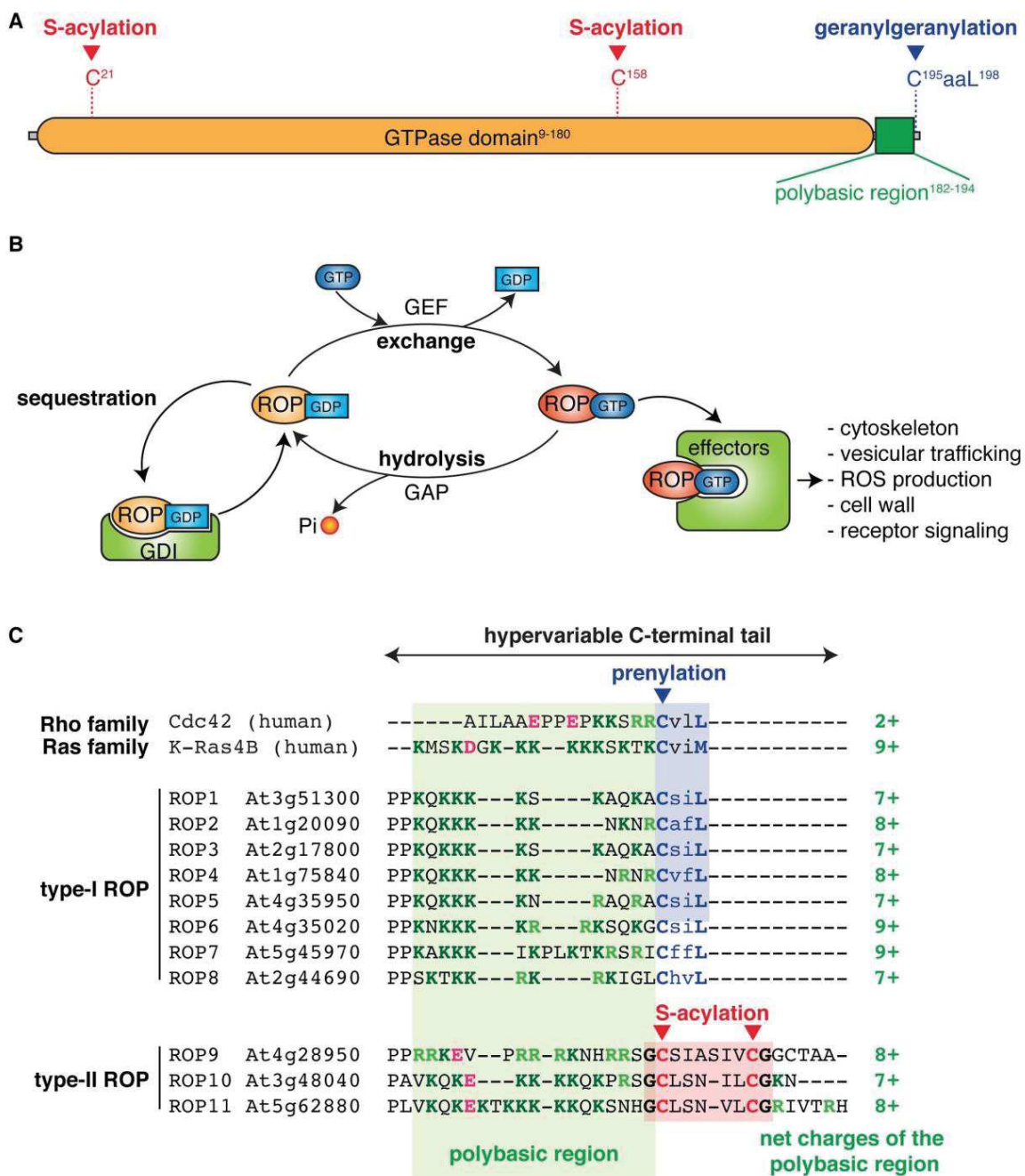


Figure 1. ROP structure and GTPase cycle. A) Schematic representation of the linear architecture of type-I ROPs. The residue number (in superscript) is given for ROP6 as an example. B) Schematic representation of the ROP GTPase cycle. C) Alignment of the C-terminal hypervariable region of the 11 Arabidopsis ROPs, showing the differences between type-I and type-II ROPs. Cationic residues are highlighted in green, acidic residues are highlighted in pink, the CaaX prenylation motif is in blue (CaaL: geranylgeranylation, CaaM: farnesylation) and the GC-CG S-acylation motif in red. For comparison purposes, the sequence of the C-terminal tail of human Cdc42 and K-Ras4B are included, as the

archetypal representative of the Rho and Ras family, respectively. ROP, Rho-Of-Plant; GEF, GTPase Exchange Factor; GAP, GTPase Activating Protein; GDP, Guanosine DiPhosphate; GTP, Guanosine TriPhosphate; Pi, inorganic phosphate; GDI, Guanosine nucleotide Dissociation Inhibitor.

Plants lack the Ras subfamily, hence Rho of plants (ROP) is the sole representative of the Ras superfamily. However, to some extent, ROPs can fulfil functions that are attributed to either Rho and/or Ras in animals. Like canonical animal Rho GTPases, ROPs are involved in signaling events that regulate the cytoskeletal organization and vesicular trafficking and thus, impact on the cell polarization and polar growth (Kawasaki et al., 2006). In addition, ROPs also relay and integrate signals downstream of receptor kinases, which is typically the function of Ras GTPases in animals. At the structural level, ROPs are also a mixed between Rho and Ras proteins. Indeed, their G domain is closely related to that of Rho GTPases. This explains that plants and animals Rho share effectors with common domains, such as the Cdc42 and Rac-Interactive Binding (CRIB) domain. However, ROP hypervariable C-terminal tail is more related to that of the archetypal Ras protein K-Ras4B, than to the Rho protein Cdc42 (see Figure 1C). Given the importance of this region on small GTPases localization (see below), this difference has a significant impact on ROP membrane dynamics, which by many accounts is closer to Ras than Rho proteins. Thus, throughout this review, and when appropriate to discuss their function and regulation, we will draw parallels between ROPs and animal Rho but also Ras proteins.

Since the fluid mosaic model proposed in the early 70s, our vision of membrane organization has become more complex (Martinière and Runions, 2013; Jaillais and Ott, 2020). Substantial studies involving advanced microscopy techniques revealed that the plasma membrane contains different domains with different biophysical properties wherein proteins and lipids involved in signaling can selectively interact with their effector molecules. The spatial organization of small GTPases into membrane domains is essential for their regulation, especially in the case of the Rho and Ras subfamilies. In plants, the impact of membrane domain formation on ROP activity is also rising. Here, we will review, the recent advances in our understanding of how membrane domains, from micrometer to nanometers size, can influence ROP signaling in plants, and to what extent the ROP spatio-temporal regulation acts in similar or different ways as in other eukaryotic systems.

ROP membrane domains

ROPs laterally segregate at the plasma membrane in so-called membrane domains. These domains can vary in size and according to the nomenclature introduced by Ott (2017) they will

be referred to microdomains when bigger than 1 μm and nanodomains when they are below that limit (Ott, 2017).

a) ROP membrane microdomains

A considerable number of ROPs isoforms can form microdomains within the membrane. These structures are bigger than 1 μm , relatively stable over time and are hallmarks of cell polarity. Therefore, they can be easily visualized with conventional fluorescence microscopy (Ott, 2017). Among some of the first polarity markers found are the auxin efflux carriers from the PIN family (Gälweiler et al., 1998). After that, a great number of proteins, and lipids were found in membrane microdomains, including the four sides and corner of root cells (i.e. rootward, shootward, inner and outer lateral polar domains), plasmodesmata, lobe and neck regions of leaf pavement cells, plant-microbe interfaces, the different regions of tip growing cells (i.e. shank, sup-apical and apical), or the sites of local cell wall modification (e.g. Casparian strip, pollen aperture, xylem pit-field, trichome) (Tapken and Murphy, 2015).

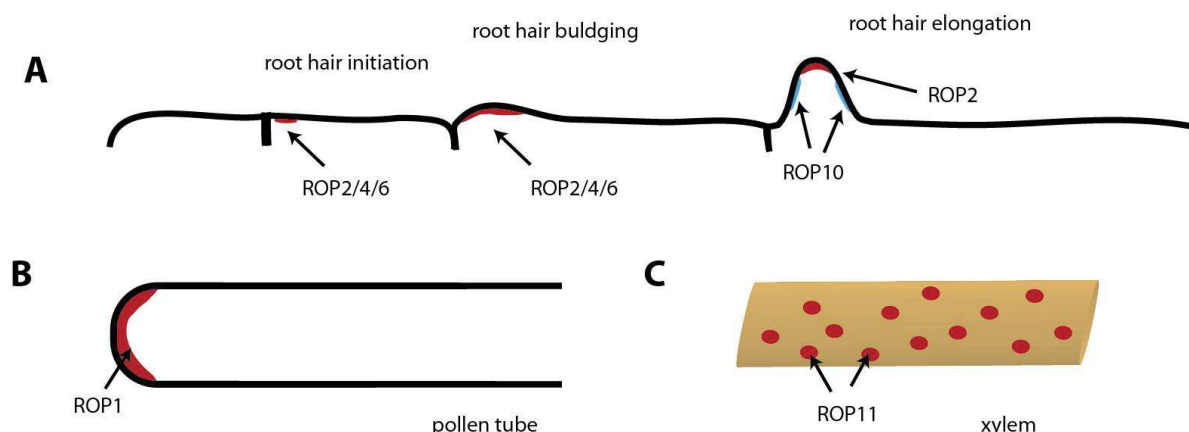


Figure 2. ROP microdomains in different cell types. A) In trichoblast cell, ROP2/4/6 accumulate in the root hair initiation domain (RHID). During hair elongation, ROP2 is present at the root hair tip whereas ROP10 accumulates at the shank. B) In the pollen tube tip, ROP1 is present in a microdomain. Its accumulation fluctuates over time during pollen tube growth. C) During xylem differentiation, ROP11 is present in microdomains that lead to the formation of cell wall pits.

ROP polarity in tip growing cells.

There are two cell types in plants that grow using tip growth: pollen tubes and root hairs. Tip growing cells are highly polarized, with distinct polar domains corresponding to the very tip (or apical part of the growing cells), the sub-apical region, and the shank of the tube/hair. Over the years, ROPs emerged as master regulators of polarized tip growth in plants, in both root hair

and pollen tube.

In Arabidopsis, ROP2, ROP4 and ROP6 localize in large microdomain (around 5-10 μm) at the so-called root hair initiation domain (Molendijk et al., 2001; Jones et al., 2002; Fischer et al., 2006; Ikeda et al., 2009; Denninger et al., 2019). This domain marks the future site where the root hair will initiate in root hair-bearing epidermal cells (i.e. trichoblast). Importantly, root hairs initiate at a constant and predictable position along the trichoblast, next, but not directly at the rootward pole of the cell (Masucci and Schiefelbein, 1994; Grebe et al., 2002; Stanislas and Jaillais, 2019). ROPs recruitment at the root hair initiation domain follows a two steps assembly: first, an initiation phase where the root hair initiation domain is predefined by GEF3 and drives ROP2/4/6 localization; then a polar growth phase, that is sustained by GEF4 (Denninger et al., 2019; Stanislas and Jaillais, 2019). The roles of the different ROP isoforms were also illustrated by the usage of constitutively active (CA, constitutively locked in the GTP-bound conformation) or dominant-negative (DN, locked in a GDP-bound conformation) ROPs. For example, both ROP4-CA and -ROP6-CA expression induces root hair swelling (Molendijk et al., 2001), and ROP2-DN results in less and shorter hairs, whereas ROP2-CA plants produce more and longer hairs than wild type (Jones et al., 2002). The same was confirmed with loss of function for *rop2*, *rop4*, *rop6* single, double or triple mutants. Hair length was reduced by about 70% in the *rop2xrop4RNAixrop6* triple line (Gendre et al., 2019). In addition to GEF3 and GEF4, the trans-Golgi network (TGN)-localized YPT-INTERACTING PROTEIN 4a and 4b are central trafficking components in ROP activation and ROP-guided root hair initiation (Gendre et al., 2019). Indeed, the secretion-defective *yip4a yip4b* mutant has decreased levels of ROP2 at the root hair initiation domain, which likely leads to shorter root hair (Gendre et al., 2019). Gendre and coworkers proposed that ROP2 can be found in the TGN (e.g. SYP61-containing vesicles), which are part of the secretory pathway and could be essential for ROP activation and polar accumulation.

The importance of the proteo-lipid environment in the establishment of the root hair initiation domain was exemplified by Stanislas and coworkers (Stanislas et al., 2015). They described that ROP2 and ROP6 accumulate together with phosphatidylinositol-4-phosphate 5-kinase 3 (PIP5K3), DYNAMIN-RELATED PROTEIN (DRP) 1A and 2B in a sterol-rich membrane domain. They uncovered that ROPs, PIP5K3, and DRPs are localized to the root hair initiation domain before root hair bulging. By contrast, D6 PROTEIN KINASE (D6PK), which serves as a modulator of this process, switches from a rootward polarity to the root hair initiation domain at the time of root hair emergence (Stanislas et al., 2015).

The cytoskeleton can also participate in ROPs membrane localization. Indeed, the

MIICROTUBULE-ASSOCIATED PROTEIN 18 (MAP18) was shown to interact genetically and physically with ROP2, controlling ROP2 localization at the root hair initiation domain (Kang et al., 2017). Moreover, the maintenance of polarized ROP in the root hair initiation domain is related to its ability to be dissociated from the membrane by GDI activity. The absence of SCN1/RHO GDI1 induces the formation of ectopic GFP-ROP2 microdomain leading to super numerate root hair initiation domains and eventually the formation of several root hairs per trichoblast (Carol et al., 2005). Oppositely, SCN1/RHO GDI1 overexpression is able to relocalize to the cytoplasm constitutive active ROP2-CA (Jeon et al., 2008). Later when the root hair starts to elongate ROP10 gets recruited to the cell shank. This atypical localization is mediated by a phosphoinositide kinases FORMATION OF APLOID AND BINUCLEATE CELLS 1 (FAB1) or its direct product phosphatidylinositol-3,5-bis phosphate PI(3,5)P₂ (Hirano et al., 2018). Together, they coordinate microtubule organization and deposition of cell wall components that contribute to the stiffness of the root hair shanks (Hirano et al., 2018).

The link between the regulation of the ROP cycle and its polarized localization in tip growing cells was also explored in the pollen tube model system (Qin and Yang, 2011). In this system, ROP1, and to a lesser extent ROP5, form a tip localized domain (Gu et al., 2005; Feng et al., 2016). Their maintenance at the tip is determined by several GAP proteins. Firstly, ROP1 ENHANCER 1 (REN1) is localized in subapical cytoplasmic vesicles and to the apical membrane (Hwang et al., 2010). As *ren1* has swollen pollen tube and enlarged ROP1 domain, it is thought to control ROP1 polar localization through a negative feedback-based mechanism (Hwang et al., 2010). More recently, another GAP, REN4 was associated with this interplay (Li et al., 2018). REN4 is mostly localized on the lateral plasma membrane region, but shows a transient tip localization. When REN4 accumulates in the pollen tube tip, the intensity of fluorescently-tagged ROP1 decreases. REN4 is controlling ROP1 membrane association by initiating its removal from the plasma membrane through clathrin-mediated endocytosis. In addition, the temporal oscillation of REN1 and REN4 at the pollen tube tip spatially controls ROP1 localization and consequently participate in pollen tube growth (Qin and Yang, 2011; Li et al., 2018). Restriction of Rho GTPase signaling to the growth site by negative regulators appears to be a common mechanism between different organisms. Examples of loss of GAPs or expression of constitutively active Cdc42 GTPase show increased growth depolarization in filamentous fungi (Ushinsky et al., 2002).

ROP microdomains in cell wall patterning.

In metaxylem cells, cell wall pits are formed thanks to GTP loaded ROP11 organized in microdomains of several tenths of micrometers. The maintenance of those microdomains

requires the coordination between the ROP11 activator, GEF4, and the ROP11 inhibitor, GAP3 (Oda and Fukuda, 2012; Nagashima et al., 2018). Interestingly, the reconstruction of evenly distributed domains can be achieved in a heterologous system, solely using ectopic expression of the three proteins (Oda et al., 2018). This reconstruction requires intact ROP11 that can cycle between its GTP-bound and GDP-bound states, as GTP-locked ROP11 induced disorganization of ROPGEF4 in membranes. In addition, ROP11 microdomains are spatially restrained by cortical microtubules through the IQ67-DOMAIN13 (IQD13) and CORTICAL MICROTUBULE DISORDERING1 (CORD1) proteins (Sasaki et al., 2017; Sugiyama et al., 2017). The ROP11 effector MICROTUBULE DEPLETION DOMAIN1 (MIDD1) is recruited within ROP11 microdomains and promotes microtubules depolymerization and inhibition of cell wall deposition in the pit area by interacting with the kinesin KIN13A (Oda and Fukuda, 2012; Oda and Fukuda, 2013). More recently, a second protein complex including BOUNDARY OF ROP DOMAIN1 (BDR), a ROP11 effector and WALLIN (WAL) was shown to promote actin polymerization. This complex stimulates cell wall deposition at the pit boundaries (Sugiyama et al., 2019). The coordination between these two signaling pathways, including the components of the cytoskeleton like actin and microtubules, with opposite effects on the cell wall growth, can ensure the establishment of specialized cell wall domains.

ROP microdomains in pavement cell shaping.

The jigsaw puzzle shape of pavement cells in the leaf epidermis serves as an exciting model to investigate the mechanisms for cell shape formation (Wasteneys and Galway, 2003). The development of Arabidopsis pavement cells are divided into three stages: Stage I starts when pentagonal or hexagonal initial cells expand along the leaf long axis to form slightly elongated polygons. These cells initiate multiple outgrowths or localized lateral expansion from their anticlinal walls into adjacent cells, producing stage II cells with multiple shallow lobes alternating with indentations or necks. As early lobes expand, reiterative lobe and neck formation continues, resulting in highly lobed interlocking cells (stage III). The cell-to-cell signaling is crucial for spatiotemporal coordination of lobe outgrowth with inhibition of outgrowth in the corresponding indented region of the adjacent cell (Fu et al., 2005a). The cytoskeleton is also implicated in pavement cell development. Cortical microtubule bundles arranged transversely in the neck regions can restrict expansion (Wasteneys and Galway, 2003). In contrast, lobe initiation and outgrowth appear to require cortical fine actin filaments localized to sites lacking well-ordered cortical microtubules (Frank and Smith, 2002; Fu et al., 2002). ROP2 and ROP6 have been described to fine-tune different parts of the cytoskeleton to achieve the jigsaw puzzle shaping of the pavement cells. ROP2 promotes the formation of

cortical diffuse F-actin and lobe outgrowth via its effector ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN4 (RIC4) (Fu et al., 2005a). In the lobe, ROP2 suppresses well-ordered cortical microtubules by inactivating another effector, RIC1 (Fu et al., 2002; Fu et al., 2005a). In the opposite neck region, ROP6 activates RIC1 to promote well-ordered microtubules and to suppress ROP2 activation (Fu et al., 2005a; Fu et al., 2009). ROP2 and ROP6 are activated by auxin, via the TRANSMEMBRANE KINASE (TMK1 to 4) leucine rich repeat receptor-like kinase LRR-RLK pathway (Xu et al., 2014). Auxin orchestrates the polarization of PIN1 that together with ROP2 and their positive feedback loop, act with the antagonizing ROP6 pathway to generate localized extracellular (or apoplastic) auxin. The activation of ROP2 and ROP6 pathways by the TMK pathway could explain how uniform concentrations of auxin leads to the establishment of cell regions that define lobe or indentation forming sites (Xu et al., 2010; Xu et al., 2014).

b) ROP membrane nanodomains

Technological advances within the field of microscopy, including total internal reflection fluorescence microscopy (TIRFM) and super-resolution microscopy, applied to plant samples have enabled more precise definition of protein organization in membranes (Konopka and Bednarek, 2008; Kleine-Vehn et al., 2011; Li et al., 2011; Li et al., 2012; Martinière et al., 2012; Hao et al., 2014; Hosy et al., 2015) . Because of the development of these powerful microscopy techniques, an increasing number of molecules have been described to be localized in domains smaller than a micron. Nanodomain-localized proteins include proteins involved in various physiological and molecular contexts, for example in auxin transport (Kleine-Vehn et al., 2011), abscisic acid signaling (Demir et al., 2013), immunity (Bücherl et al., 2017; McKenna et al., 2019) and many more.

Recently, the role of protein nanodomain organization during cell stimulation was exemplified in the case of ROP signaling. Platre and coworker 2019 showed that ROP6 is recruited in nanoclusters of 50 to 70 nm, only a few minutes after auxin stimuli in Arabidopsis root cells (Platre et al., 2019). Constitutive active ROP6 forms nanodomain in absence of any cell stimulation, suggesting that ROP6 nanodomain concentrates GTP bound ROP6. In a preprint article, ROP6 appear to be also nanoclustered upon osmotic stimuli (Smokvarska et al., 2020). GTP-bound ROP6 is remobilized in clusters together with its effector RESPIRATORY BURST OXIDASE HOMOLOG (RBOH). This enzyme is responsible for ROP6 dependent ROS accumulation upon sorbitol treatment. Whereas ROP6 variants that cannot be redirected into clusters show no increase in ROS accumulation upon activation, ROP6-CA expressing plants

display high ROS and numerous nanodomains without any cell stimulation. More importantly, the RBOH recruitment in nanodomains is constitutive in this condition, suggesting that activated ROP6 can meet or recruit RBOH in specific plasma membrane nanodomains (Smokvarska et al., 2020).

It seems that the nanoclustering of ROPs is a general feature for many developmental and adaptation processes. Regrouping of active GTPase in nanoclusters is a shared feature in yeast and animals, including K-Ras, Rac1, and Cdc42 (Prior et al., 2003; Remorino et al., 2017; Meca et al., 2019). The mechanism behind the formation of the ROP membrane domain is complex and regulated by many different factors in parallel. It thus remains partially understood. But some of the common underlying principles for the formation of polar domains and nanodomains are emerging, including self-organization of the ROP/ROP-GEF/ROP-GAP module, lipid modifications and lipid interactions.

Domain formation and maintenance

a) Domain self-organization by reaction-diffusion system

Recent studies proposed a novel ROP membrane domain organization via self-organization processes, where overall order arises from local interaction between components of a disordered system (Figure 3A). Reaction-diffusion processes also called activator-inhibitor systems are based on two chemical species where one is a local activator with a slow diffusion and the other one creates a global inhibition from its fast diffusion (Figure 3A). This creates a spontaneous periodic pattern of the two entities (Figure 3B). Reaction-diffusion systems are involved in various tissue and organ development. In the field of small GTPase, it was described in yeast that the Cdc42 regulates polarity through a reaction-diffusion mechanism (Goryachev and Pokhilko, 2008). Cdc42 is activated by its GEF, Cdc24, and this system provides positive feedback due to the recruitment of Cdc24 by activated Cdc42. The reaction components (Cdc42 and Cdc24) diffuse to the cytoplasm of small intracellular pits which leads to domain competition. This results in a single budding domain in yeast (Kozubowski et al., 2008; Wu et al., 2015).

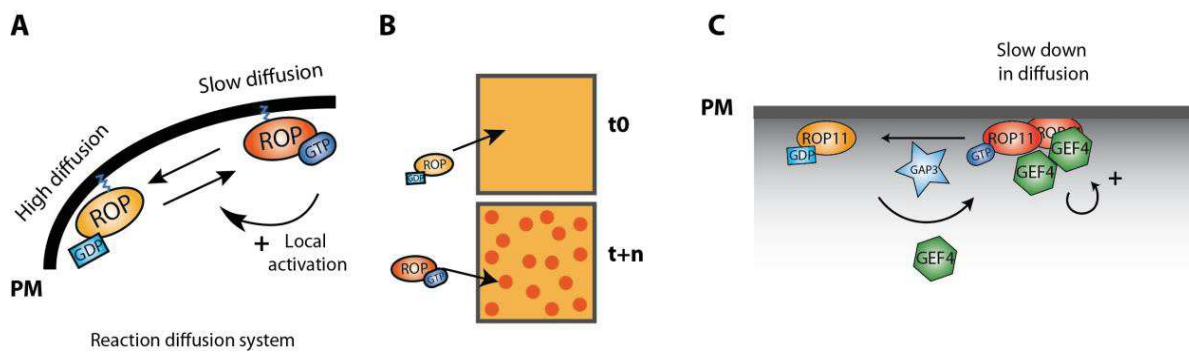


Figure 3: ROP self-organization through the reaction-diffusion system. A) Two chemical species (here ROP-GDP and ROP-GTP bound) are under reaction-diffusion process, when a long-range inhibition by a higher diffusion inhibitor and a local activation by lower diffusion of activator exist. B) The diffusion of the inhibitor (ROP-GDP) and activator (ROP-GTP) creates spontaneously periodic spatial distribution. C) In the case of cell wall pit formation, ROP11 is globally inhibited by the cytoplasmic GAP3. It gets activated by GEF4 that decreases its diffusion. As GEF4 makes dimers, it induces a local recruitment of new ROP11 molecules.

In Arabidopsis metaxylem cells, ROP-activation/inactivation cycle can generate the ROP11-activated microdomains. This would not be reachable without feedback from GEF and slow diffusion of the GEF-ROP complex. In the formation of cell wall pits, the density of ROP-activated microdomains is determined by levels of GEFs (GEF4 in this case) and GAPs (GAP3), suggesting that GEFs and GAPs determine the pattern of pits by regulating the underlined ROP-activated microdomains pattern (Nagashima et al., 2018). Unlike in yeast, in metaxylem vessel cells, the ROP-activation cycle leads to co-existing multiple domains depending on the levels of GEFs and GAPs to direct pitted cell wall patterns (Nagashima et al., 2018). When GEF4 interacts with ROP11, ROP11 diffusion decreases. As GEF4 exists as dimer, it induces a local recruitment of new ROP11 protein (Figure 3C) (Nagashima et al., 2018). In line with this study, Hwang et al., 2010 described that GAP1 and GDI1 act in maintaining the active ROP1 domain to a proper level and size during rapid continuous pollen growth. Overexpression of GAP1 or GDI1 suppresses the enlargement of the active ROP1 cap induced by ROP1 overexpression. Once ROP1 is inactivated by GAP1, it is probably removed from the pollen tube apex membrane into the cytosol by GDI1. Therefore, the global downregulation of ROPs signaling might be linked to the negative feedback needed in the self-organization system (Hwang et al., 2010).

Finally, different from this self-organization principle of reaction-diffusion is the proposed

regulation for the root hair initiation domain. In contrast to the multiple cell wall pits of the metaxylem, the positioning and establishment of a single polar root hair at a stereotypical position within the trichoblast plasma membrane is likely to require a mechanism that eliminates randomness during its formation. GEFs have been proposed to play a key role in this non-random polarization. GEF3 mediates the recruitment and temporally-controlled ROP activation, followed by later recruitment of GEF4, which gives a higher order of domain organization (Denninger et al., 2019). A similar mechanism was described in *Drosophila* embryos, where the GEF Dizzy and the heteromeric GEF complex ELMO-Sponge are required for polarization of the GTPase Rap1 (Bonello et al., 2018). What determines the selection of a single ROP activation domain in this case is still an open question. Besides GEFs, other players, such as the membrane lipid composition, are essential to establish the root hair initiation domain (Stanislas et al., 2015). Furthermore, it is likely that GEFs are themselves locally recruited and/or regulated by membrane receptor kinases (Duan et al., 2010; Stanislas and Jaillais, 2019), which could act as positional anchors in connection with the cell wall and extracellular signals (Jaillais and Ott, 2020).

b) ROP lipid modifications

As mentioned previously ROP triggers signaling when targeted to the plasma membrane and membrane binding is ensured by posttranslational lipid modifications. Based on their amino acid sequences, ROPs are composed of an N-terminal catalytic G-domain where nucleotide and effector binding take place and a C-terminal hypervariable domain, which is responsible, at least in part, for subcellular targeting (Figure 1A). This C-terminal is composed of Cys motifs and Arg-Lys-rich polybasic regions (Figure 1C). ROP1 to ROP8 belong to a group of ROPs called Type-I ROPs where their hypervariable region contains a CaaL motif (Figure 1C). The cysteine can be modified by isoprenyl lipid geranylgeranyl on the 20C (Feiguelman et al., 2018). The geranylgeranylation of type-I ROPs is required for membrane interaction and mutations in the CaaL motif, which prevents the lipid modification, also abolishes membrane interaction (Figure 4A) (Sorek et al., 2017). Type-II ROPs (ROP9 to ROP11) lack the CaaL motif and have instead a GC-CG motif linked by five or six aliphatic residues and undergo S-acylation by the C16 palmitate or C18 stearate fatty acids via labile thioester bond (Figure 1C) (Wu et al., 2011). Like for type-I ROPs, lipid modification on the C-terminal tail of type-II ROP is required for membrane targeting (Lavy and Yalovsky, 2006). The adjacent polybasic region is responsible for membrane attachment and interaction with certain membrane lipids (see the

section below on lipids).

In addition to their modification in the C-terminal hypervariable region, some ROPs can undergo activation-dependent S-acylation in residues inside the G-domain. Indeed, it was found that constitutively active ROP6, but not inactive ROP6 is acylated in this region with palmitic and stearic acids (Figure 1A) (Sorek et al., 2017). Moreover, a recent preprint suggests that auxin treatment, which activates ROP6 (Xu et al., 2014), also leads to ROP6 S-acylation as show in a preprint paper (Pan et al., 2019). Interestingly, ROP6 acylation was linked to membrane partitioning (Sorek et al., 2010). Whereas wild-type ROP6 is present in detergent-resistant membranes and soluble membrane, the constitutively active ROP6-CA is mainly in detergent-resistant membranes and point mutations in the two cysteines in the G-domain that are acylated abolish this association (Sorek et al., 2010). In addition, ROP6-CA acylation mutant (ROP6-CA C21S/C158S) cannot recapitulate the classical ROP6-CA overexpression phenotype on root hair polar cell growth, endocytosis uptake, and ROS accumulation, suggesting that nanodomain targeting is important to achieve the ROP6 function (Sorek et al., 2010). The role of acylation on ROP6 nanodomain formation was observed directly by super-resolution microscopy approach recently. Indeed, whereas ROP6 nanoclustering increase upon hyperosmotic stimulus, the point mutated mEOS2-ROP6 C21A/C158A stay diffusible and do not cluster after cell stimulation (Figure 4B) (Smokvarska et al., 2020). The plasma membrane localization of this acylated mutant was similar to that of the wild-type ROP6 (Figure 4A). This indicated that proper acylation of ROP6 is dispensable for plasma membrane targeting but essential for its localization in nanodomain. Whereas the mutation C21A/C158A does not interfere with the GTPase activity of ROP6, they abolished the downstream signaling like osmotically induce ROS production (Sorek et al., 2010; Smokvarska et al., 2020). ROP2 and ROP10 were also described to undergo S-acylation (Lavy and Yalovsky, 2006; Wan et al., 2017). As C158 is conserved among many type-IROP, S-acylation seems to be a general feature of ROP signaling (Sorek et al., 2010; Sorek et al., 2011; Sorek et al., 2017). How transient S-acylation upon activation is regulated and triggers ROP nanodomain formation and maintenance is an outstanding question.

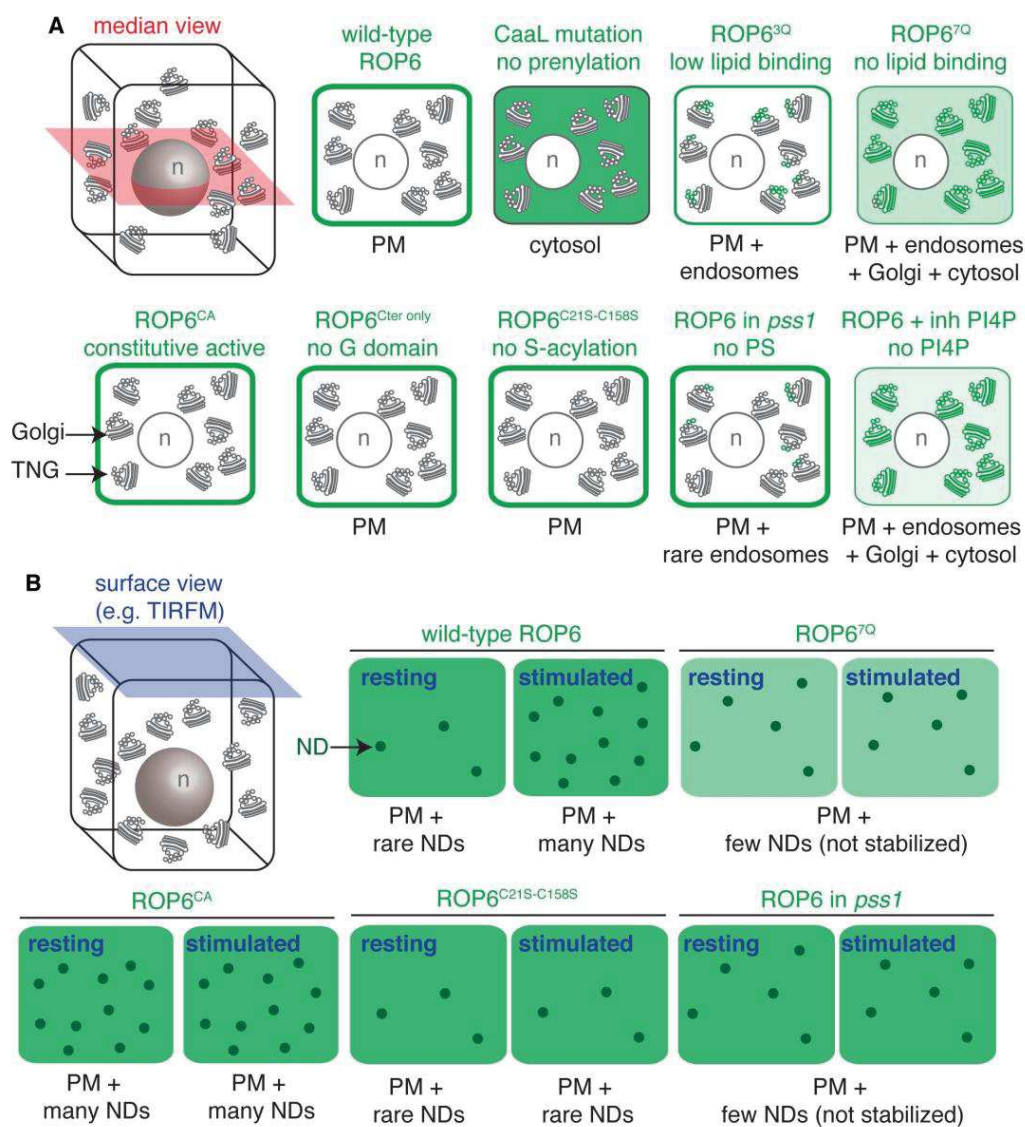


Figure 3: Summary of ROP6 or ROP6 mutant localization depending on the background and activation status. Schematic representation of xFP-ROP6 (where x can be different fluorescent protein) localization as seen by A) confocal microscopy (median view), and B) TIRF microscopy (surface view). The ROP6 mutant versions or the background/treatment are indicated on top. In each condition, the localization is indicated in green, with a dark shade of green indicating strong accumulation, while a lighter green indicates weaker accumulation. In B, “Resting” indicates the ROP6 localization in the absence of treatment, while “stimulated” indicates ROP6 localization following auxin treatment or osmotic stress. PM, plasma membrane; ND, nanodomain; inh PI4P, inhibition of PI4P synthesis using a pan-PI4Kinase inhibitor; *pss1*, *phosphatidylserine synthase1*; PS, phosphatidylserine; G domain, GTPase domain; n, nucleus. Note that “lipid binding” refers to interaction with anionic lipids via the ROP6 polybasic C-terminal tail.

Protein S-acyl transferases, or PATs, are integral membrane proteins that catalyze the addition of fatty acyl groups to cysteine residues within proteins and they have an Asp-His-His-Cys (DHHC) motif (Batistič, 2012). In Arabidopsis, there are 24 PATs. However, only the substrates of PAT4 and PAT10 have been identified (Zhou et al., 2013; Wan et al., 2017). PATs operate by a two-step process. First, the Cys residue of the DHHC motif is auto-acylated by binding an acyl group, such as palmitate or serate. Following this, the acyl group is transferred to a Cys residue in the target protein (Hou et al., 2009; Mitchell et al., 2010; Jennings and Linder, 2012). The cysteine in the DHHC motif is the auto-S-acylation site because a mutation in this residue results in loss of auto-acylation of PAT10 (Qi et al., 2013). PAT4 was documented to genetically interact with ROP2 and potentially acylates it. Indeed in *pat4-2*, ROP2 localization to the plasma membrane is significantly weaker. This is a similar observation as for ROP2^{C20S/C157S}, in which the two potential S-acylation cysteine residues were mutated into serine (Wan et al., 2017). The mislocalization of ROP2 from the plasma membrane in absence of PAT4, although significant, is not drastic, which indicates that PAT4 is not essential for the membrane/cytoplasm partitioning, unlike prenylation, but a putative role in nanodomain formation remains open (Chai et al., 2016). In Arabidopsis nine out of 24 PATs are located at the plasma membrane, the rest reside in the endoplasmic reticulum (ER) and the Golgi apparatus (Batistič, 2012). This is different from mammalian and yeast PATs which are mainly localized to the Golgi (Rocks et al., 2010) and might suggest that regulation of proteins by PATs at the plasma membrane may be more important or could drive membrane subcompartmentalization in plants.

S-acylation is a transient lipid modification, suggesting that Acyl Protein Thioesterases (APT) could also participate in the organization of ROP partitioning at the plasma membrane (Tabaczar et al., 2017; Kathayat et al., 2018). APT is able to cleave the thioester linkage between the fatty acid and cysteine sulfhydryl. Unlike in animals and fungi, in plants little is known about the de-S-acylation process. Medicago MtAPT1 is the first protein thioesterase with de-S-acylation activity that has been found in plants (Duan et al., 2017). More recently, maize ZmB6T1C9 was proposed as S-acyl protein thioesterase but these data are based solely on structural homology to human acyl-protein thioesterases 2 (APT2) (Bürger et al., 2017). To date, there are no reports describing the mechanism of ROP de-S-acylation.

c) Anionic lipids

Electrostatic interactions with anionic lipids are required for ROP plasma membrane targeting.

Both type-I and type-II ROPs contain in their hypervariable C-terminal end a polybasic region adjacent to the lipid anchor modification site (Figure 1C). This region is made of a lysine- and arginine-rich stretch and has a net positive charge that varies from +7 to +9 (Figure 1C) (Platre et al., 2019). Proteins from the Ras/Rho super-family in animals and yeasts also contain a polybasic region, which is known to interact with anionic phospholipids, likely via electrostatic interactions (Heo et al., 2006; Platre et al., 2019). Anionic phospholipids possess a negatively charged head group, and within the endomembrane network, they mostly correspond to phosphatidylinositol and their phosphorylated derivative the phosphoinositides (e.g. phosphatidylinositol-4-phosphate (PI4P) or phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂), phosphatidic acid and phosphatidylserine (Noack and Jaillais, 2017). The presence of a polybasic region in ROP C-terminus suggests that they also likely bind to anionic phospholipids. Accordingly, ROP6 interacts with all anionic phospholipids *in vitro* and these interactions are fully abolished upon the mutation of the ROP6 polybasic region (conversion of seven lysine/arginine residues into neutral glutamine, ROP6^{7Q}, which has only two remaining positive charges) (Platre et al., 2019). *In vivo*, a minimal construct consisting of a fusion between the yellow fluorescent protein mCitrine and ROP6 C-terminal tail (polybasic region and CaaL motif) is sufficient to recapitulate ROP6 plasma membrane localization (Figure 4A) (Platre et al., 2019). In addition, the transgenic expression of mCitrine-ROP6^{7Q} shows that these mutations do not fully abolish ROP6 interaction with membranes, but rather impair the nature of the membrane it is targeted to. Indeed, ROP6^{7Q} has a much wider localization than wild-type ROP6, being targeted to the plasma membrane, but also to intracellular compartments (Figure 4A) (Platre et al., 2019). Thus, the interaction between the ROP6 polybasic region and anionic phospholipids likely specifies ROP6 targeting to the plasma membrane, rather than membrane interaction itself. This result is consistent with the notion that ROP6 interacts with anionic lipids through electrostatic interactions. Indeed, the inner leaflet of the plasma membrane is the most electronegative cytosol-facing membrane of plant cells and thus membrane proteins containing polybasic regions with seven-net positive charges or higher tend to be specifically localized to this compartment (Simon et al., 2016). Accordingly, a ROP6^{3Q} mutant, which has six-net remaining positive charges, shows an intermediate localization, with increased plasma membrane targeting compared to ROP6^{7Q} but which is still present in intracellular compartments, by contrast to wild-type ROP6 (Figure 4A) (Platre et al., 2019). The high electrostatic field of the plasma membrane is mainly powered by PI4P, with a more modest contribution of phosphatidic acid and phosphatidylserine (Simon et al., 2016; Platre et al., 2018). Pharmacological inhibition of PI4P synthesis triggers the

relocalization of wild-type mCitrine-ROP6 into intracellular compartments, which mimics the localization of mCitrine-ROP6^{7Q} (Figure 4A) (Platre et al., 2019). Thus, ROP6 polybasic region is required for the specific localization of ROP6 at the plasma membrane, a process that is PI4P dependent.

ROP interactions with phosphoinositides contribute to their polar localization.

Given that all ROPs have polybasic regions of similar net positive charges, it is likely that most ROPs will interact with anionic lipids and rely on the plasma membrane electrostatic properties for proper targeting. For example, ROP2 is enriched at the root hair initiation domain together with PI(4,5)P₂ and a PI4P 5-kinase (PIP5K3) (Kusano et al., 2008; Stenzel et al., 2008; Stanislas et al., 2015; Denninger et al., 2019). In addition, type-II ROPs also require their polybasic region for plasma membrane targeting (Lavy and Yalovsky, 2006). In the case of type-II ROPs, a minimal construct containing only the C-terminal tail (polybasic region and GC-CG S-acylation motif) is efficiently targeted to the plasma membrane, while deletion of the polybasic region leads to the solubilization of this minimal construct (Lavy and Yalovsky, 2006). Thus, the polybasic regions of type-II ROPs could be required for membrane interaction *per se* and not only to provide targeting specificity to the plasma membrane, like for the type-I ROPs, ROP6 (Lavy and Yalovsky, 2006; Platre et al., 2018). In addition, the type-II ROP, ROP10, interacts with PI(3,5)P₂ and the PI3P 5-Kinase FORMATION OF APLOID AND BINUCLEATE CELLS1 (FAB1) (Hirano et al., 2018). ROP10 accumulates in the shank of root hairs together with FAB1 and PI(3,5)P₂, in a complementary domain to the tip-localized ROP2/4/6, PIP5K3 and PI(4,5)P₂ (Kusano et al., 2008; Stenzel et al., 2008; Hirano et al., 2018; Stanislas and Jaillais, 2019) (Figure 2A). ROP10 in the shank is required for root hair morphology likely by regulating microtubules dynamics in this domain. Together this suggests that different ROP isoforms may interact with specific anionic lipids and/or anionic lipid metabolic enzymes to control their polarity.

Phosphatidylserine stabilizes ROP6 in plasma membrane nanodomains.

Phosphatidylserine is also involved in plasma membrane electrostatics, albeit to a lower extent than PI4P and phosphatidic acid (Platre et al., 2019). A mutant in the PHOSPHATIDYLSERINE SYNTHASE1 (PSS1) gene, which completely lacks phosphatidylserine, has little impact on ROP6 plasma membrane targeting (Figure 4A) (Platre et al., 2019). However, the *pss1* mutant is impaired in many ROP6-mediated cellular and

developmental responses, including auxin-induced inhibition of endocytosis. This suggests that phosphatidylserine could be required for ROP6 function but not plasma membrane localization. Interestingly, super-resolution single-molecule imaging showed that ROP6 nanoclustering in response to auxin was abolished in the *pss1* mutants or for the mutated ROP6^{7Q} version (Figure 4B) (Platre et al., 2019). Together, these results suggest that ROP6 nanoclustering in response to auxin is required for downstream signaling. As mentioned earlier, ROP6 directly interacts with phosphatidylserine via its polybasic region. Accordingly, phosphatidylserine itself is clustered in nanodomains in the plasma membrane, and these nanodomains colocalize with ROP6-induced nanoclusters following auxin treatment (Platre et al., 2019). In addition, in the context of leaf pavement cells, a recent preprint suggests that auxin-induced ROP6 nanoclustering is also dependent upon membrane sterols, which are involved in ROP6 activity in this cell type (Pan et al., 2019). However, it remains to be explored whether sterols are themselves required for the formation of phosphatidylserine-containing nanodomains.

- d) Lipid modification and lipid interaction is a multistep process contributing to ROP localization.

Taken together, ROP proteins appear to contain multiple and complex localization information. Indeed, we propose that proper ROP membrane targeting is a multistep process involving successive lipid modification and lipid interactions, at least for type-I ROPs (Figure 5). First, type-I ROPs are soluble proteins synthesized in the cytosol. They are then prenylated (geranylgeranylation) at the surface of the ER, which provides hydrophobic anchoring to membrane bilayers. This lipid anchor is sufficient to ensure membrane attachment but on its own, it does not provide membrane specificity. Second, in the absence of additional targeting sequences, ROPs can explore many different compartments within the endomembrane network with equal probability (Simon et al., 2016; Platre et al., 2019). This exploration is likely dependent on membrane trafficking as demonstrated by the key role of the TGN-mediated pathway in ROP localization in root hair forming cells (Gendre et al., 2019). Third, the polybasic region engages in electrostatic interactions with the negative charges of anionic phospholipids (Platre et al., 2019). The TGN is the most electrostatic intracellular compartment within plant cells (Platre et al., 2018). This could favor the collection of ROPs at the TGN for their subsequent delivery to the plasma membrane, which could explain, at least in part, the key role of this compartment for ROP localization (Gendre et al., 2019). Note that such a

“collect and delivery” system has not been formally demonstrated in plants but has been proposed for human K-Ras (Figure 5) (Schmick et al., 2014; Schmick et al., 2015). Fourth, the plasma membrane is enriched in anionic lipids compared to the membrane of other compartments (Simon et al., 2016), which allows to kinetically trap ROPs to the plasma membrane in a PI4P-dependent manner (Platre et al., 2019). Fifth, upon activation (e.g. auxin, osmotic stress, genetic CA mutation), ROP6 is S-acylated at the plasma membrane (Sorek et al., 2010; Pan et al., 2019). S-acylated proteins tend to localize in regions of the membrane that are locally more ordered (Jaillais and Ott, 2020). S-acylation could thus act as a trigger to relocalize ROPs into plasma membrane nanodomains upon activation, which could contain sterols (Figure 5) (Sorek et al., 2010; Pan et al., 2019; Smokvarska et al., 2020). Seventh, these nanodomains are enriched in phosphatidylserine, which directly interacts with ROPs and may stabilize them in these membrane domains (Platre et al., 2019). This model has mostly been investigated with ROP6. However, it is likely that it can be extended to other type-I ROPs, at least to some extent. In the case of ROP6, plasma membrane targeting and nanocluster formation are both required for downstream signaling. An outstanding question in the field is why ROP6 (and possibly other ROPs) need to be localized in nanodomains to be competent for signaling? While this is still largely an open question, we discuss some hypotheses in the section below on the functional importance of ROP nanodomains.

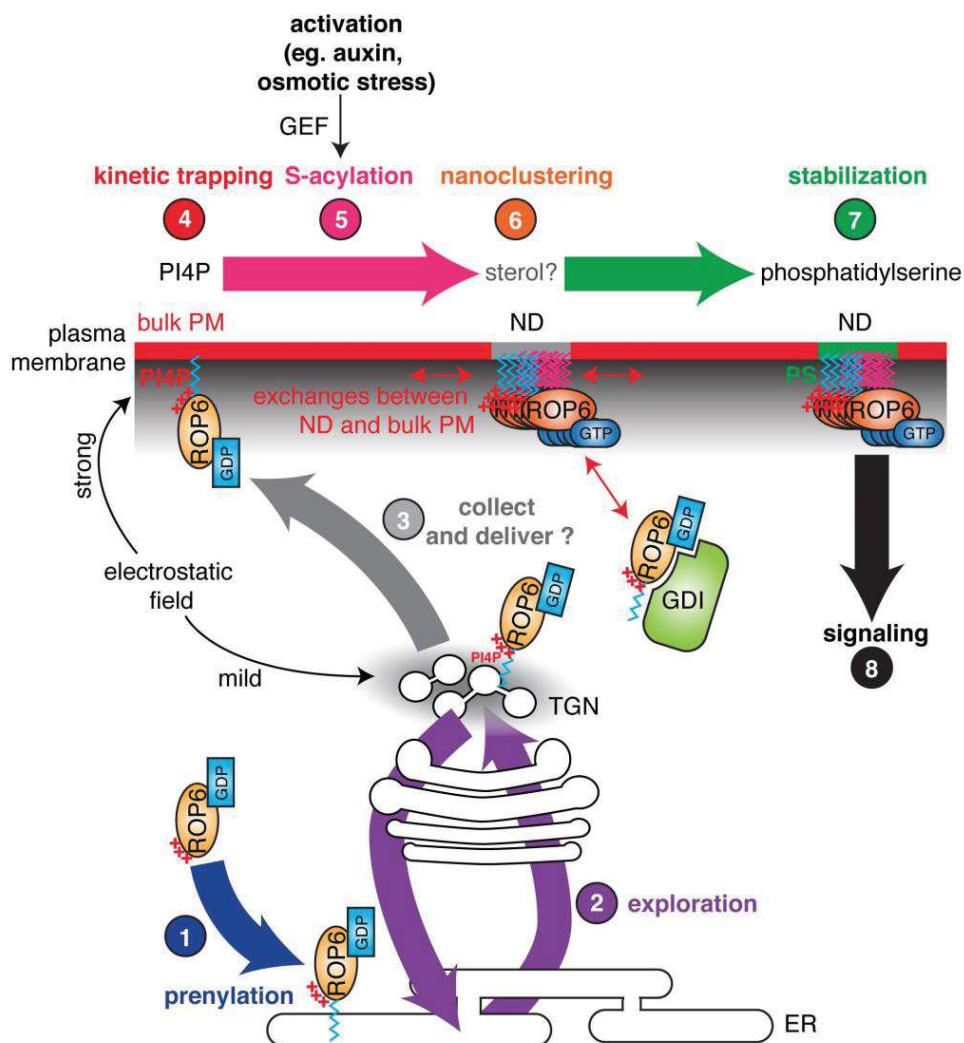


Figure 5: A multistep model for the role of lipid modifications and lipid interactions in the regulation of ROP6 localization and function. Note that this is a hypothetical model with some of the steps that have not been formally demonstrated (e.g. step 3). The exact order in which the events at the plasma membrane unfolds is also currently unknown and this scenario represents one possibility among others. It is, for example, possible that nanoclustering and stabilization are concomitant or that interaction with phosphatidylserine is required upstream of S-acylation. PS, phosphatidylserine; PI4P, phosphatidylinositol-4-phosphate; ND, nanodomain; GEF, GTPase Exchange Factor; GDP, Guanosine DiPhosphate; GTP, Guanosine TriPhosphate; GDI, Guanoside nucleotide Dissociation Inhibitor

Effect of nanodomains on signaling parameters

a) Specific effector recruitment for a nanodomain specialization in single cells

As described throughout this review, there are numerous examples where one ROP isoform is involved in different signaling pathways. For example, ROP6 is mediating pavement cell and root hair growth, ROS production, auxin-mediated inhibition of endocytosis, lateral root development, salicylic acid-regulated pathogen response (Molendijk et al., 2001; Fu et al., 2005b; Fu et al., 2009; Sorek et al., 2010; Poraty-Gavra et al., 2013; Venus and Oelmüller, 2013; Wang et al., 2015). These signaling processes often happen in different cell types, and even in different organs, but contrasting this, studies have also placed ROP6 downstream of different stimuli in the exact same cell type (Platre et al., 2019; Smokvarska et al., 2020). Auxin and osmotic signals act differently on cells by having opposite effects on endocytosis (Chen et al., 2012; Lin et al., 2012; Zwiewka et al., 2015; Martiniere et al., 2019) Whereas, ROP6 is mandatory for auxin-mediated endocytosis inhibition, osmotic signal trigger membrane internalization through ROS accumulation (Chen et al., 2012; Lin et al., 2012; Zwiewka et al., 2015; Martiniere et al., 2019). One question that emerges from these findings is how multiple signal stimuli can generate specific responses through the same transducer. In membranes, both osmotic and auxin signaling induce similar size (around 50 nm) and density of ROP6 nanodomains, but their composition might differ (Platre et al., 2019; Smokvarska et al., 2020). Indeed, Smokvarska et al. show that GDP bound ROP6 colocalized and interacted in nanodomains with the effector protein RBOHD in nanodomain during osmotic stimulation. When cells are exposed to auxin, RBOHD is absent of ROP6 nanodomain suggesting that depending on the upstream signal ROP6 nanodomain composition is different (Smokvarska et al., 2020) (Figure 6D). Other ROP effectors have a plasma membrane localization that depends on their interaction with ROP (Figure 6B and 6C). It is the case for RIC1 that acts as an actin severing protein and that needs a fully functional CRIB domain to be targeted to the membrane at the pollen tip growing zone (Zhou et al., 2015). From these different results, we could speculate the existence of signal specific domains where upstream activators such as receptors and/or GEFs coexist before the activation of ROP6 and the recruitment of signal specific effectors (Figure 6D, 6G). This constitutes a tempting working model to reconcile the multifaceted roles of ROP in membrane cell signaling.

b) Time in nanodomains regulate ROP signaling

Of note, the presence of ROP6 in nanodomains was not fully abolished in the *pss1* mutant, and only the induction of ROP6 nanoclustering after auxin treatment was affected (Platre et al., 2019) (Figure 4B). This provided a unique opportunity to compare the dynamics of ROP6 inside nanodomains in wild-type plants or *pss1* mutants. Fluorescence recovery after photobleaching experiments showed that GFP-ROP6 is highly stable within nanodomains in the wild-type situation (no exchanges between the bleached and unbleached regions for >60 s). By contrast, GFP-ROP6 is not stabilized in nanodomains in *pss1* (exchanges between the bleached and unbleached areas observed within seconds). This result suggests that ROP6-phosphatidylserine interaction is not required to trigger the relocalization of ROP6 into nanoclusters but rather to stabilize ROP6 within these structures (Platre et al., 2019). Because ROP6 signaling is compromised when it cannot interact with phosphatidylserine (i.e. *pss1* mutant or expression of ROP6^{7Q}), we speculate that not only ROP6 nanoclustering is required for function but also the stabilization of ROP6 in these structures through time. This suggests that confining ROPs into nanodomains not only has a spatial function but that there is also a temporal aspect of this confinement (Figure 6H). For example, we can speculate that regrouping ROP6 into nanodomains, allows ROP6 to spatially meet its effectors (Figure 6B and 6C), but that it also favors their long-lasting interactions, which could be required for downstream signaling (Figure 6H). Furthermore, it is possible that the enzymatic reactions catalyzed by some of the downstream effector proteins are slow and thus could require long-lasting interactions with activated and clustered ROPs. In addition, increasing the dwell time of ROP6 in nanodomains could impact the local diffusion of cytosolic cortical effector components. In animals, such local slowdown in diffusion has been shown to impact phase separation of cortical proteins (Case et al., 2019; Huang et al., 2019). The induction of phase-separated condensates in the cytosol, just below plasma membrane nanodomains, could provide local conditions that are favorable for downstream enzymatic reactions (Figure 6F) (Jaillais and Ott, 2020). Although this hypothesis remains untested at the moment, it is consistent with the idea that nanoclustering of activated ROP6 is not sufficient to trigger signaling and that ROP6 needs to remain stable in these nanodomains to trigger a downstream effect. Whether membrane-less phase-separated organelles exist below the plasma membrane and in conjunction with plasma membrane nanodomains is an outstanding question for our future understanding of ROP signaling in particular and membrane organization in general.

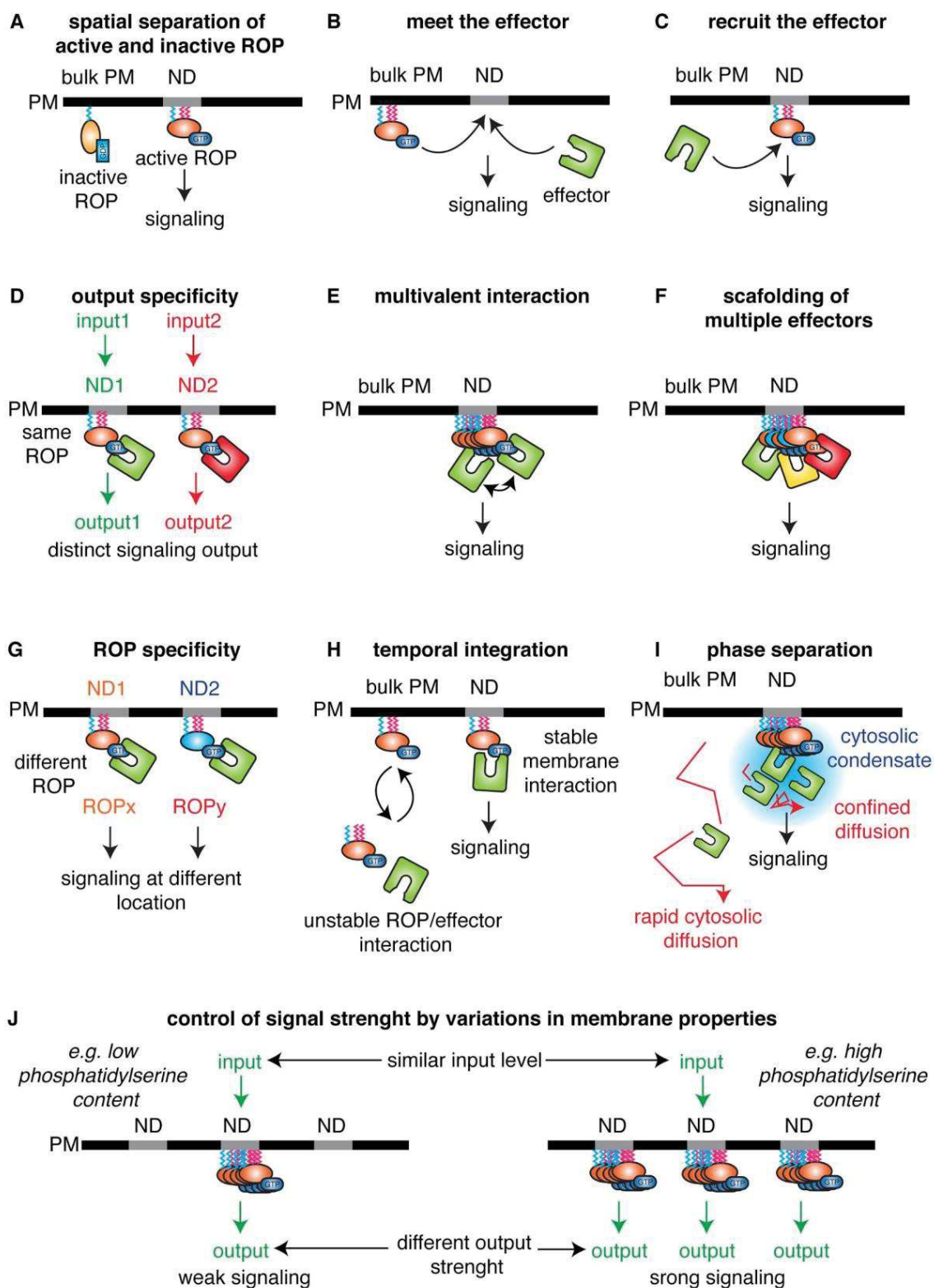


Figure 6: Possible scenarios for the function of nanoclustering in ROP signaling. Note that most of these scenarios have not been formally demonstrated and that they remain to be explored. In addition, these scenarios are not mutually exclusive and not exhaustive. A) Nanodomains appear to spatially segregate active (GDP-loaded) and inactive (GTP-loaded) ROPs. However, only a portion (~30 to 40%) of activated ROPs are localized in nanodomains and this model does not explain by itself why nanodomains are required for downstream signaling. B) Nanodomains could be a favorable membrane environment for ROPs to meet with their effectors, explaining the requirement for nanodomains in signaling. C) ROPs may also recruit their effectors specifically in nanodomains to induce signaling. D) Nanodomains may act as signaling platforms to trigger specific output, as seen in response to auxin treatments or osmotic stress (Smokvarska 2020, Platre 2019). E) It is possible that ROP nanodomain formation allows the clustering of downstream effectors, which could induce low energy interactions between them. Such multivalent interaction within nanodomains could be required for signaling and therefore explains the requirement for nanoclustering in signaling. F) It is possible that multiple ROPs cluster within the same nanodomains, thereby scaffolding several ROP effectors required for signaling. G) It is also possible that multiple ROPs localize to different nanodomains, which could ensure the propagation of specific cytosolic outputs. H) The stabilization of ROPs in nanodomains could increase their dwelltime at the plasma membrane, which may be required to stabilize their effector at the cell surface and/or allow them sufficient time to catalyze their reaction. I) The clustering of ROPs in nanodomains may induce the formation of phase-separated cytosolic condensates below the plasma membrane, which could be required for effector function. J) The clustering of ROPs in nanodomains may quantitatively control the strength of the output signal even when the input signal is constant. According to this model, variation in membrane lipids could act like a rheostat that tunes the signaling capacity of cells. Such a model has been proposed for the membrane lipid phosphatidylserine during the ROP6 response to auxin (Platre et al., 2019). PM, plasma membrane, ND, nanodomain. Effectors are represented as a U-shaped protein.

Conclusions and perspectives.

Being highly compartmentalized, the plasma membrane can control multiple aspects of cell signaling. This is exemplified by ROP proteins. Indeed, a substantial number of publications have revealed the formation of domains of large size in the plant plasma membrane (higher than 1 μ m); including at the site for root hair initiation, in the tip of pollen tube or in metaxylem cells (Figure 2). These microdomains determine the recruitment of ROP effector proteins in specific parts of the cell, allowing polar growth or formation of cell wall pits in xylem cells. More recently and thanks to super resolution microscopy, it was revealed that ROP can also form domains at a nanometric scale. These nanodomains are necessary to trigger downstream cell

signaling, but how they quantitatively control ROP signaling remains a mostly open question. We proposed a non-exhaustive and non-mutually exclusive list of potential mechanisms by which ROP6 nanoclustering could impact the recruitment, dynamics, and activation of effectors at the membrane (Figure 6).

There are a number of similarities between micro and nanodomains, which suggest that microdomains could be a higher-order organization of nanodomains. This idea is supported by the fact that the lipid environment of microdomains and nanodomains presents some similarities. For example, in the root hair initiation domain, ROP2 and ROP6 accumulate in sterol rich membranes (Stanislas et al., 2015). ROP6 is associated with detergent resistant membrane and its clustering and diffusion is sensitive to beta cyclodextrin that depletes sterol from membranes (Sorek et al., 2010; Pan et al., 2019). The mechanism under action for the formation of microdomains and nanodomains could also be similar. For instance in budding yeast, polar localization in microdomains of active cdc42 is the result of reaction-diffusion processes driven by a small number of molecules that regulate its GTPase cycle (Chant and Herskowitz, 1991; Wedlich-Soldner et al., 2004; Goryachev and Pokhilko, 2008). In plants, a similar hypothesis was proposed for ROP11 microdomains in cell wall pits of metaxylem cells. In addition, mass-conserved activator-substrate models have shown that some parameters of the GTPase cycle, like the saturation point for active Rho, can themselves control switching between the unipolar and multipolar domains in cells (Chiou et al., 2018). Therefore, the control of GTPase cycle by GEFs, GAPs and GDIs might control nanodomains formation in plant cells.

Root hair formation nicely exemplifies the fact that different ROP isoforms could accumulate in distinct microdomains within the same cell. Indeed, whereas ROP2 is localised at the tip of growing hair, ROP10 is observed in cell shanks (Hirano et al., 2018). This opens the questions whether the same is true for nanodomains: can different ROPs localize to similar or different nanodomains within the same cells or even within the same polar patch (Figure 6G)? For the moment, only ROP6 was demonstrated to form nanodomains, but this property is probably shared with other ROP isoforms. It is possible that ROPs will cluster in the same nanodomains and potentially scaffold multiple effectors to trigger specific outputs (Figure 6F) or they could accumulate in distinct nanodomains, which could ensure specificity (Figure 6G). This is a particularly intriguing question since ROPs have only little variations in their amino acid sequences, including conserved acylation sites in the core of the protein and a polybasic

sequence at their C-terminus that contains between 7 and 9 positive charges (Figure 1C). Only, their C-terminal lipidation changes between type-I ROPs that are prenylated and type-II ROPs that are acylated (Figure 1C). However, work on K-Ras suggests that subtle variations of its C-terminal tail, for example a simple lysine-to-arginine substitution, which does not change its overall net positive charge, may induce interaction specificities for different anionic lipids (Zhou et al., 2017). This suggests that interactions with anionic lipids may not be purely electrostatics and that subtle variations in ROP C-terminal tail may change their anionic lipid specificity. This is an outstanding question to explore in the future.

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3.2.3 ROP6 regulation by guanine nucleotide exchange factor (GEFs)

3.2.3.1 Introduction

ROP6, as being crucial and necessary for the osmotic signaling can segregate in nanodomain. Interestingly, very similar behavior was described for auxin signal where ROP6 also forms stimuli dependent nanodomains. How a single ROP can encode for several signals is still unknown. One possible hypothesis might be due to upstream activator which differs between the different signals. Because the ultimate goal of my thesis is to understand how plants perceive and transduce osmotic signal, we have looked at proteins that might come upstream of ROP6 and act as potential activators. Due to their direct roles in the activation of ROPs, we focused on GEFs, while keeping in mind that the activation of ROPs can also take place by inhibition of GAPs or GDIs (Fig1-B from review p.65).

As described previously, GEFs are a plant-unique family, with some members containing PRONE catalytic domain (Berken et al., 2005; Gu et al., 2006). The specificity for ROPs is likely associated with the presence of a small Gly residue in the insert regions of ROPs (134 in ROP4) at a position that, in nonplant ROPs, is occupied by a large Arg residue (Thomas et al., 2007; Berken and Wittinghofer, 2008). The PRONE domain is an auto-active catalytic domain inhibited by its C-terminus (Berken and Wittinghofer, 2008). The possible mechanism is that the latter can be phosphorylated during their activations, making the PRONE domain accessible to ROPs proteins (Huang et al., 2013; Zhang and McCormick, 2007). In *Arabidopsis* there are 14 GEFs, in addition to SPIKE1 (SPK1) which shares a homology with CZH-type mammalian GEFs (CDM-zizimin homology) (Gu et al., 2006; Basu et al., 2008). SPIKE1 in the root is necessary for the activation of ROP6 during auxin signaling (Lin et al., 2012). In fact, the loss-of-function phenotype of SPIKE1 is similar to the phenotype of the *pin2* mutants, i.e. a greater density of lateral roots and loss of gravitropic responses. By activating ROP6, SPIKE1 allows the maintenance of the distribution of PIN2 and thus ensures the correct localization of auxin in the root tip (Lin et al., 2012). However, SPK1 is also involved in the activation of ROP2 and ROP4 to organize the network of cortical microtubules to inhibit anisotropic growth in the petal (Ren et al., 2016). Besides SPK1, other GEFs also interact with ROP6. Particularly in the development of root hair, it has been reported that GEF4 and GEF10 interact with ROP6 which has been demonstrated by BIFC and by a 2YH (Huang et al., 2013). *gef4* and *gef10* compromised the RH initiation and elongation by disturbing the ROP signaling resulting from a decreased level of activated ROP in the double mutant *gef4gef10*. Studies in

RH also have demonstrated that ROPGEFs function downstream of the CrRLK FERONIA (Duan et al., 2010; Yu et al., 2012; Huang et al., 2013). Thus, the activation of at least some ROPGEFs depends on intercellular signaling that involves phosphorylation by membrane-bound RLK1Ls. For more precise description about the regulation of different GEFs in the ROP signaling see the review.

How and which GEF activates ROP6 during osmotic treatment is still an open question at this stage. By a reverse genetic, I tested a series of GEF loss of function plants for their role in osmotically induced ROS production. Some of the GEFs can already serve as potential candidates (GEF4 and GEF10) since they were described to interact with ROP6. Since ROP6 is involved in another signaling pathway like auxin, I wanted to test whether or not the identified GEFs could act as a general activator of ROP6 or an osmotic specific regulator.

3.2.3.2 Results

GEF14 is necessary for the osmotically induced ROS accumulation

First potentially interested candidates from the GEF family that we tried were GEF4 and GEF10 as they are reported to interact with ROP6 and the double *gef4gef10* has lower levels of activated ROP that compromises root hair initiation. The quantification of ROS was assessed with the DHE dye. Osmotic treatment induces a significant increase of DHE fluorescence for both control plant (Col-0), *gef4* and *gef10* mutants (Fig11-A). This result shows that GEF4 and GEF10 is not needed for the osmotically induced ROS production. Therefore we continued searching for other possible GEFs. To be able to exercise its function of regulator, the potential GEF candidates must be expressed in the same cells as ROP6. Besides GEF4 and GEF10, GEF2, GEF3, GEF11 and GEF14 are also expressed in the same cell types as ROP6. We tested single loss-of-function mutants *gef 2-1*, *gef3-1*, *gef11* on control or hyperosmotic stress conditions (Fig11-B). It seems that these single mutants are responsive to osmotic stimuli in a similar manner than the Col-0 plants. Therefore, the proteins GEF2, GEF3 and GEF11 are not involved in osmotic signaling.

However, absence of the osmotically induced ROS is noted in two loss-of-function alleles of GEF14 (Fig11-C). It would therefore seem that the accumulation of ROS during the osmotic signal is dependent on GEF14. Nevertheless, it remains possible that some redundancy may exist between isoforms, as GEFs are known to form heteromers (Nagashima et al. 2018). In this line, we tested the triple and quadruple loss-of-function mutant *gef 1/4/10* and *gef 1/4/10/14* (Waadt, and Schroeder, 2016). These plants grow normally but are known to have altered ABA signaling (Waadt, and Schroeder, 2016). While for *gef 1/4/10* a ROS induction is

observed, for *gef 1/4/10/14* osmotic treatment does not induce ROS accumulation in cells (Figure 11-D- work by Lucille Gorgues (Master 2 in 2020). This confirms the role of GEF14 in the production of osmotically induced ROS. *gef 1/4/10* has a higher level of ROS induction, and this might derive from a miss regulation of GEF14. Thus, among the GEFs tested, it seems that GEF14 is specifically necessary to induce ROS during an osmotic signal. Therefore, we can hypothesize that GEF14 is the single activator of ROP6 during the osmotic signal (among the GEF mutant tested).

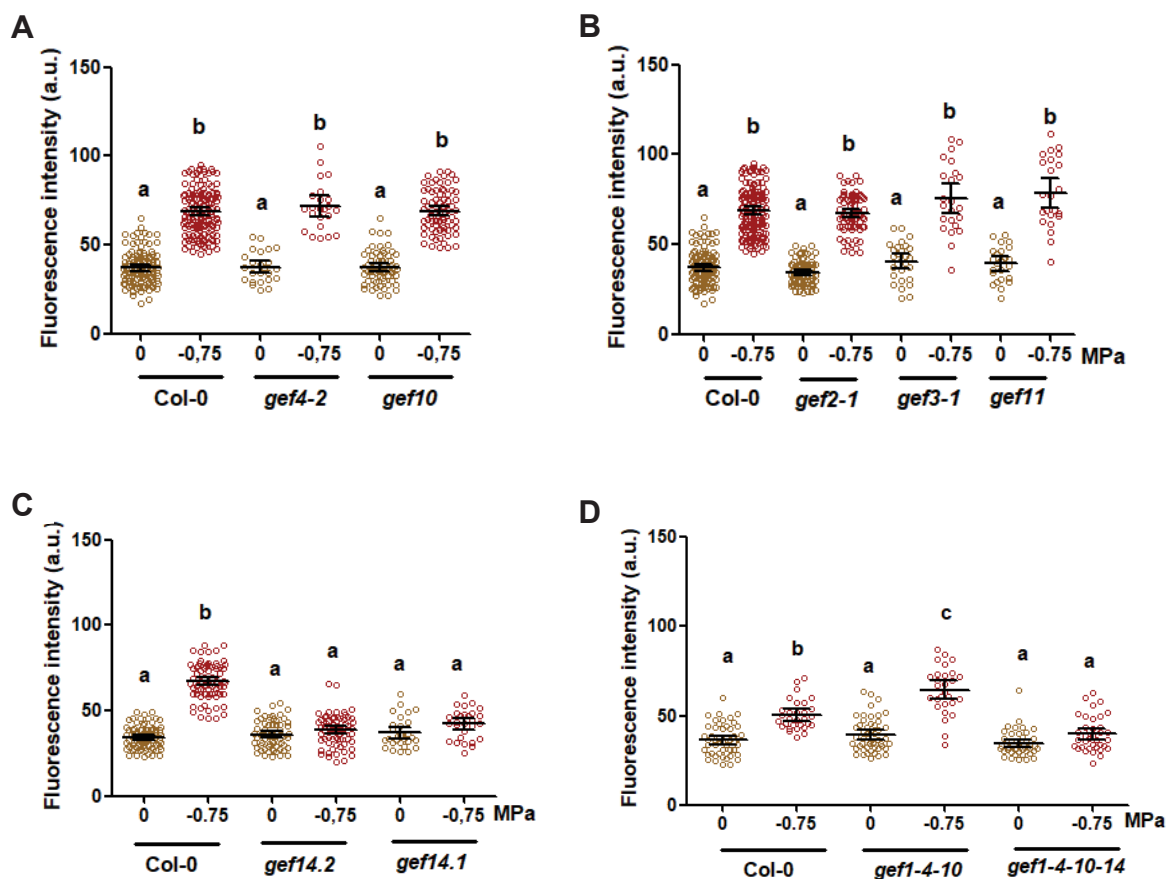


Figure 11. **Involvement of other GEF isoforms in the osmotic signaling response**

Quantification of dihydroethidium fluorescence (DHE) signal for the Col-0 genotype and single mutants *gef 4-2* and *gef10* (A), *gef2-1*, *gef3-1*, *gef11* (B); two alleles of GEF14, *gef14-1*, *gef14-2* (C); triple *gef1/4/10* and quadruple *gef1/4/10/14* (D) in control condition and after 15 minutes of hyperosmotic stress. Note that data from D was generated by L.Gorgues (Master 2-2020)

The error bars correspond to the confidence interval at 95%. The classes are calculated after an ANOVA and a Tukey test. They show the significant differences between the mean values (p -value < 0.05). The data represent the sum of three independent biological replicates.

GEF14 might code for osmotic signal specificity

As explain before, we know that ROP6 is involved in signaling pathways other than the osmotic (Sorek et al., 2010, Poraty-Gavra et al., 2013, Wang et al., 2015, Platre et al., 2019). Because we hypothesize that GEF14 is the activator of ROP6 during osmotic signal, we wonder if it could extend to other signaling pathways that go through ROP6 as well.

First, we tested if some other signaling pathways that induce ROS accumulation in cells might go through ROP6. Therefore, we used flg22 and ABA treatment. The flg22 is a well describe peptide derive from bacterium flagellum that induces RbohD activation and the accumulation of ROS in cells. The phytohormon ABA was described to induce RbohF and in smaller extend RbohD. *rop6.2* mutant, unlike the WT plants and the complemented line, show insensitivity to these signals (Fig12-A). This result suggests that ROP6 acts as a signaling hub in the cells as it is needed for hormonal signaling (auxin and ABA), abiotic stress (osmotic signal) and biotic stress (flg22). To test whether GEF14 is specific for the osmotic signal, the *gef14.1* mutant was incubated with flg22 and ABA. In both cases, an increase in the ROS signal could be detected although it was slightly lower in the case of stimulation with ABA (Fig12-B). For comparison, other GEF mutants were tested. Mutants of GEF2 and GEF10 show an increase in ROS after stimulation with flg22 and ABA although in the latter case they are slightly weaker than the control (Fig12-C). The data seems to indicate that GEF14 is specifically involved in the activation of ROP6 in osmotic stress environment.

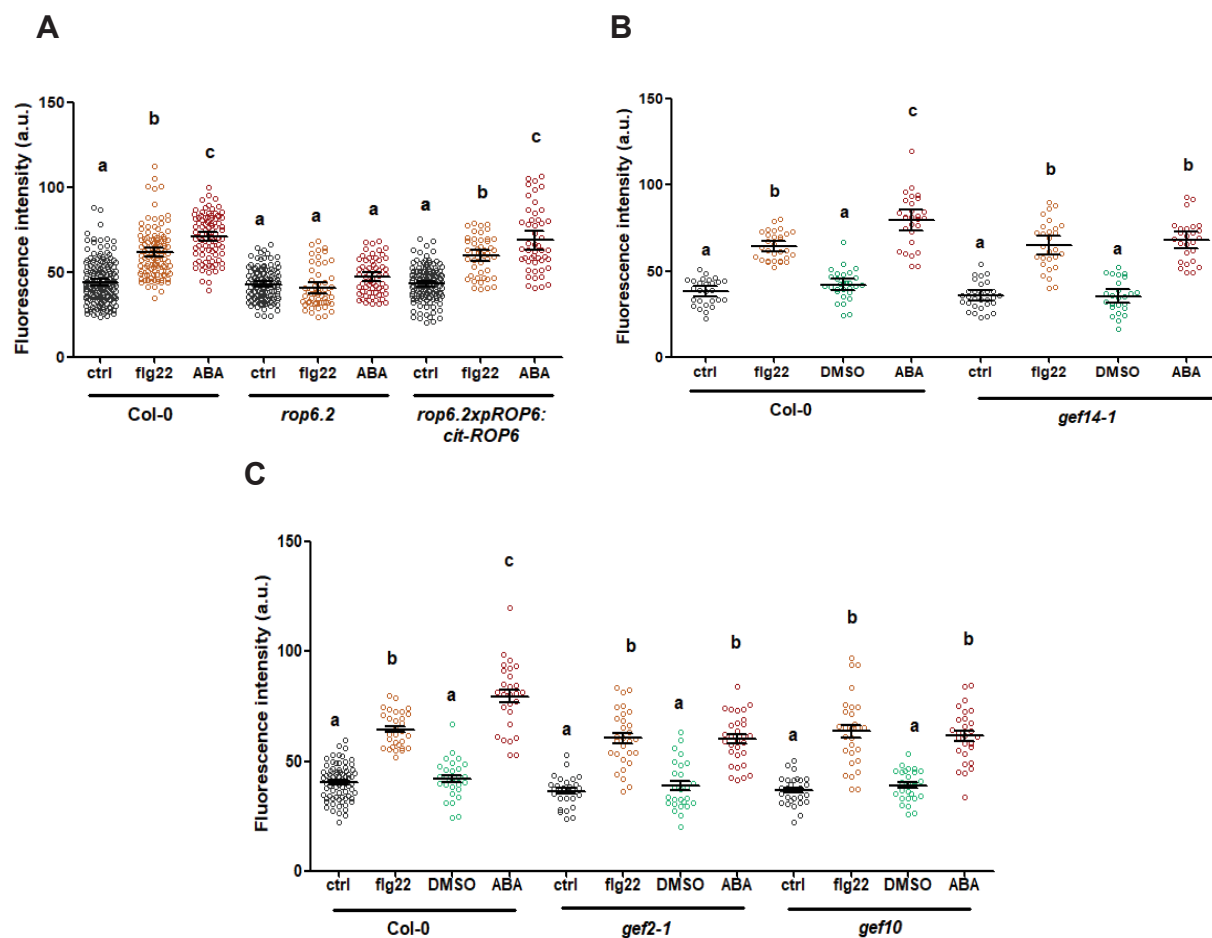


Figure 12. Implication of ROP6 and different GEFs in the response to flg22 and ABA

- A) Quantification of the fluorescence of dihydroethidium (DHE) in the root cells of the control genotype Col-0, *rop6.2* and of a complemented line *rop6.2xpROP6: Cit-ROP6* in control condition or under treatment with either 1 μ M flg22 for 30 minutes, or 1 μ M ABA for 1 hour.
- B) Quantification of the fluorescence of dihydroethidium (DHE) in the root cells of the control genotype Col-0 or *gef14-1* in control condition or under treatment with either 1 μ M flg22 for 30 minutes, or 1 μ M ABA for 1 hour.
- C) Quantification of the fluorescence of dihydroethidium (DHE) in the root cells of the control genotype Col-0, *gef2-1* or *gef10* in control condition or under treatment with either 1 μ M flg22 for 30 minutes, or 1 μ M ABA for 1 hour.

The error bars correspond to the confidence interval at 95%. The classes are calculated after an ANOVA and a Tukey test. They show the significant differences between the mean values (p -value < 0.05). The data represent the sum one biological replicate.

3.2.3.3 Discussion

The responses of plants to osmotic stress have been widely characterized; however the mechanisms of signal perception and transduction are poorly understood. We identify a small GTPase ROP6 that is essential for the induction of ROS-like secondary messengers as well as the integrated response of plants to the osmotic signal. Although ROP6 plays a key role, the actors of its activation during this signal remain unknown. GEFs are ideal candidates for this function because they allow the GTPase shuttling from GDP-off to GTP-on state. Our loss-of-function approach demonstrated that GEF14 is involved in the accumulation of ROS during the osmotic signal and also that this isoform would be specific for this signal. These results contribute to a better understanding of the osmotic signal in plants, but above all allow a better understanding of how small GTPase signaling is set up at the level of the cell membrane.

Is GEF14 a specific upstream of ROP6 in the osmotic signaling cascade?

Interestingly enough, the expression of GEF14 isoform is induced during salt stress, which is known to induce osmotic signaling in plant (Shin et al., 2009). This transcriptional regulation suggests a feedforward loops on ROP6 regulation by stress. In addition, we have highlighted a potential specificity of this isoform. That is, the tested single mutants of GEF2, GEF3, GEF4, GEF10, GEF11 but also the triple mutant *gef1-4-10* show induction of ROS during the osmotic signal (Fig11). Interestingly, GEF4 and 10 are known to interact with ROP6. Thus, it is tempting to think that GEF14/ROP6 pathway encodes for osmotic signal whereas GEF4 or GEF10/ROP6 pathways will devoted to other type of signals. Indeed, ROP6 is involved in other signals transduction such as auxin, ABA and flg22. We have shown that plants disturbed in GEF14 gene were responding to flg22 and ABA stimuli (Fig12-B). However, it is possible to observe reduced ROS accumulation compared to control upon stimulation with ABA. This phenomenon is also observable with GEF2 and GEF10. Multiple mutants of GEF (*gef1/4/10* and *gef1/4/10/14*) have previously been reported to be hyposensitive to ABA. This results in reduced inhibition of primary root elongation and germination (Zhao et al., 2015). It would therefore be interesting to carry out other tests for the ABA signaling, in particular testing all the mutants of GEFs including the triple and quadruple line. Indeed, it is not excluded that only some of the GEF participate to the ABA induce ROS production. In addition, it was shown that ABA induces the relocalization and later the degradation of GEF1 through CPK4 phosphorylation (Li et al., 2018). If this model could be extended to other GEF isoform, it could represent an interesting feedback pathway to reduce ROS accumulation upon ABA treatment. In any cases, we should keep in mind that loss of function approach can have some limitation. For instance, only gain-of-function plants have shown distinct phenotypes for these two GEFs.

GEF4 is sufficient for root hair extension while GEF10 is sufficient for root hair initiation. Also overexpressing line GEF3 can form RHID in atrichoblastic cells, such as hypocotyl (Denninger et al., 2019). This is why a gain of function approach would allow us to know whether GEF14 is sufficient for the accumulation of ROS during the osmotic signal. But, we will also keep in mind that most of the GEFs exist as auto-inhibited form.

How GEF14 can encode for the osmotic specific signal.

ROP6 nanodomain formation is essential to transduce different signal in plant cell. Thus, nanodomains are not by themselves able to encode for signal specificity. But, we have found that ROP6 nanodomain composition change between auxin signal and osmotic signal, Rboh protein been recruited in ROP6 nanodomains only after osmotic stimulation (Smokvarska et al., 2020). A possibility is that GEF14 regulate the ROP6/Rboh complex formation; and in general the specificity of GEF14 for the osmotic signal and the potential role of other GEFs for ABA, auxin and flg22 signals, would be spatial segregation within the membrane. Thus, there might be specific nanodomains for each signal in which the GEFs involved are located. The recruitment of ROP6 would therefore take place in different domains, which would participate in the specificity of the downstream cellular response. More detailed discussion of this aspect is in the general discussion and perspectives section (page 162).

How GEF14 is activated by the osmotic signal?

We have developed a model of the regulation of ROP6 during an osmotic signal that depends on GEF14 which would be its activator. An important question is how GEF14 get activated. As explained previously, the C-terminal has an auto inhibitory function on the PRONE catalytic domain. It is therefore possible that an activator of GEF14 is protein responsible for the phosphorylation of the C-terminal making GEF14 active. Studies have already demonstrated interactions between GEFs and Receptor like kinases (RLK1Ls). This is the case with GEF1 which forms a complex with ROP1 and PRK2, a receptor protein kinase (Li and Liu, 2012). PRK2 has been shown to phosphorylate GEF1. In addition to this kinase, it has also been shown that GEF4 and GEF10 are located downstream of FER, a known CrRLK (Huang et al., 2013). Co-IP experiments followed by identification by mass spectrometry could be a good choice for trying to fish out the possible kinase. We could use several triggers such as osmotic stimuli, ABA or flg22. Because we believe that the GEF14-ROP6 interaction is osmo-specific, treating with other stimuli would be good controls to remove interactions that are not osmo-specific. Thus, it would be possible to identify the regulator (s) of GEF14 and possibly ultimately lead to the perception systems of this signaling pathway.

4. Chapter 2. Interplay between cell wall sensing and osmotic signaling

The first chapter has shown that osmotic sensing in plant is mediated through small GTPase. We have described two elements of this pathway. The small GTPase ROP6 and a putative activator, GEF14 that likely confer some signal specificity. We found that this signaling module is needed for several plant responses to osmotic stress.

In this chapter 2, we will try to identify the upstream mechanism that activate GEF14/ROP6 pathway. This system might be one of the osmotic sensing machinery. In the recent years, new mechanisms explaining how plant senses their cell wall status have been described. Because, we know that osmotic signal impact cell wall/plasma membrane continuum (see introduction), I have tested the potential link between osmotic signaling and plant cell wall sensing pathways.

4.1 INTRODUCTION

Under high osmotic stress, the cell wall deforms and dissociates from the plasma membrane. The partial or complete loss of the interaction with the plasma membrane has a large effect on the wall and wall-associated proteins, which are anchored to the plasma membrane and are important for maintaining cell wall function. Osmotic stress disrupts the cellulose synthesis due to the depolymerization of microtubules under these conditions. It also affects the biosynthesis of new wall materials by regulating the localization of proteins involved in the deposition of wall components, especially in growing cells. This illustrates the crucial role of plant cell wall as a one of the first cell component reacting to osmotic signal.

Plant cell wall

The plant's striking architecture, comprised of the large surfaces required for efficient photosynthesis at a low metabolic cost, is a result of two essential attributes: large vacuole allowing accumulation of water and solutes and rigid cell wall. The cell wall is primarily made of carbohydrates with few amounts of proteins, but most importantly it is highly heterogeneous and complex structure. Once the cell growth is completed a secondary cell wall is deposited, which can give cell strength and be impermeable. But besides the numerous developmental and physiological the cell wall has its role in, one very important feature, as being at the cell "border" is its involvement in many signaling networks.

The primary cell wall is a robust structure consisting of partially redundant but interdependent polysaccharide and protein networks. Typical components of the cell wall include cellulose, non-cellulosic, and pectic polysaccharides, proteins, phenolic compounds, and water.

Cellulose micro fibrils, with their extreme tensile strength, are the main load-bearing elements of the cell wall. Xyloglucan (XG) is the main type of hemicellulose, which depending on the chain length can cross-link micro fibrils and reinforce the cell wall (Takeda et al., 2002; Whitney et al., 2006). Each cellulose microfibril is composed of β -1,4-linked glucan chains synthesized at the cell surface by cellulose synthase (CESA) complexes (CSCs), which are highly mobile membrane proteins (Paredes et al., 2006; McFarlane et al., 2014; Kieber and Polko, 2019). The ability of xyloglucan to intertwine with cellulose is limited to sites at which cellulose microfibrils are in close contact with other microfibrils, thereby forming 'biomechanical hotspots' (Park and Cosgrove, 2012a,b) that confer strength and stability to the wall (Zhao et al., 2014). An analysis of higher plant CESA protein sequences suggests that they can be categorized into six main classes (Carroll and Specht, 2011; Kumar and Turner, 2015b). Three of these classes, CESA1, CESA3, and CESA6, are responsible for producing cellulose in the primary cell walls (Desprez et al., 2007; Persson et al., 2007), while the other three classes, CESA4, CESA7, and CESA8, make cellulose in secondary cell walls (Taylor et al., 2003). However, a recent analysis of CESA proteins from the moss *Physcomitrella patens* suggests that separate CESAs are required for both primary and secondary cell walls, but these CESA proteins do not form separate classes and it seems likely that a single CESA class is sufficient to make cellulose (Norris et al., 2017). Identification of several mutants, impaired in the function of different CESA proteins, were identified. *rsw1* carries a mutation in *CESA1* that causes a temperature-sensitive radial cell expansion defect and a cellulose deficiency in all cell types investigated (Arioli et al., 1998). In this mutant, moreover, the cellulose synthase rosette complex disappears on images of freeze-fractured plasma membranes. *irx3* or *isoxaben resistant 3* is mutated in *CESA7* and shows a collapsed xylem phenotype and a greatly decreased cellulose content specifically in secondary cell walls (Taylor et al., 1999). Thus, these genes appear to encode functionally specialized isoforms required for cellulose synthesis in primary or secondary walls. *Procuste1* (*prc1*) mutant is a null allele for the CESA6 complex with similar phenotypes as those seen in *rsw1*. Scheible et al., 2001 demonstrated that mutations in CESA6 (and CESA3) confer resistance to isoxaben, an inhibitor of cellulose deposition. Because isoxaben causes radial swelling of roots, this indicates that CESA3/6 probably also contributes cellulose to primary walls. KORRIGAN1 (KOR1) is a membrane-bound endo-1,4- β -d-glucanase that is also required for cellulose synthesis (Nicol et al., 1998). GFP-KOR1 expressed in the *kor1-1* mutant background under the control of its endogenous promoter is found in discrete particles at the plasma membrane in the same cells as GFP-CESAs (Crowell et al., 2009). BIFC assays in tobacco leaves indicated that KOR1 can interact

with CESA1 and it is involved in the synthesis of glucan chains and/or their assembly into micro fibrils and intracellular trafficking of cellulose synthase complexes (Crowell et al. 2009). Moreover, XG is not the only load-bearing network. Indeed, *xxt1/xxt2* mutants, which entirely lack XG, have only subtle growth phenotypes and walls with mechanical properties comparable to those of the wild type (Cavalier et al., 2008). Pectin can also interact in vitro with cellulose. In situ interactions between cellulose and pectin was shown by a 3D nuclear magnetic resonance and showed that pectin and XG mechanically behave as a single entity (Dick-Perez et al., 2011), perhaps through covalent pectin-XG links (Popper et al., 2008). Three classes of pectin can be distinguished based on two different backbone configurations: homogalacturonan (HGA), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II). Recently, Haas et al 2020 showed that nanofilaments of pectin homogalacturonan in the cell wall can actively shape the cell and its turgor pressure independently. Mutants affected in pectin synthesis show pleiotropic phenotypes. For example, the Arabidopsis *emb30* mutants are affected in a gene that presumably functions in the secretory pathway (Shevell et al., 2000). They have a defective cell wall with abnormal localization of pectin but not of xyloglucan. In tomato, the *cnr* fruit ripening mutant is affected in the maturation process of HGA in the middle lamella of the fruit pericarp, leading to reduced cell cohesion (Orfila et al., 2001). *quasimodo1* (*qua1-1* and *qua1-2*), that are dwarfed plants and show 25% reduction in galacturonic acid levels which leads to reduced pectin levels and have deformed cell shape (Bouton et al., 2002). Analysis of Arabidopsis *mur1* mutants, which are deficient in GDP-I-Fuc, showed that their small growth is caused by the absence of fucosyl residues on RG-II, which affects its ability to dimerize through the formation of boron diester cross-links (O'Neill et al., 2001). This finding demonstrated that cell wall pectic organization is important in controlling plant growth. Other abundant non-cellulosic polysaccharides include xyloglucan, β -1,3:1,4-glucan, xylan, mannan, and callose, which fulfill various roles in mechanical support, reserve storage and development.

In addition to the polysaccharide network, structural proteins also play an important role in cell wall architecture, and typically constitute approximately 10% of the wall of growing cells. Among the structural proteins, extensins are defined as extracellular, basic, hydroxyproline (Hyp)-rich structural glycoproteins with alternating hydrophilic (X-Hypn) and hydrophobic motifs that frequently carry tyrosine residues as potential cross-linking sites (Lampart et al., 2011). Mutant analysis shows an essential role for certain extensins in primary cell wall assembly, for example the lack of EXT3 leads to embryo lethality and incomplete cell plates. (Cannon et al., 2008). More recently, extensin domain protein was also associated to

perception for cell wall integrity. In summary, all the above described elements of the cell wall need to be carefully monitored and controlled by the cell to ensure proper cellular functioning. One very important aspect of the cell's tight regulation over the cell wall is the regulation of its integrity.

Cell wall integrity (CWI)

A key element of plant's plasticity seems to involve a mechanism monitoring functional integrity of the cell wall and initiating compensatory responses when cell wall integrity (CWI) is impaired. CWI impairment is caused by cell wall damage (CWD), which can arise during exposure to biotic or abiotic stress, or simply development (Wolf et al., 2017, Novaković et al., 2018, Bacete et al., 2018). Wall constituent deficiency, disruption of the wall crosslinking, free wall fragments can all cause CWD. For example, pathogen-derived enzymes break down cell walls, which release cell wall-derived fragments. This could lead to cell wall weakening, deformation, and displacement of the cell wall relative to the plasma membrane and can eventually result in cell bursting due to the high turgor pressure of the cell (Lorences et al., 1994; Miedes et al., 2014) The cell wall fragments, such as cellobiose or oligogalacturonides (OGs; fragments of pectic polysaccharides), can activate plant immune responses (Ferrari et al., 2014; Azeve do Souza et al., 2017). Although OGs are detected through wall-associated receptor kinases (WAKs), the receptors for cellobiose have not been identified. Mechanosensitive systems may also be activated by CWD that compromises the structural integrity of the cell wall. In addition to the enzymatic actions of pathogens and mechanical damage caused by breakage or grazing, defects in cell wall biosynthetic processes can also cause CWD by preventing the production of load-bearing structural elements (Tateno et al., 2015). In line with our work it was proposed that the cell wall integrity components are acting upstream of the small GTP-ases (Huang et al., 2012), therefore it constitutes a promising axis to follow.

The cell wall damage or perturbation of the CWI can be directly monitored by cell wall sensors (Fig13). CrRLK1Ls are a group of receptor-like kinases (RLK1Ls) with two extracellular malectin-like domains that are involved in the CWI maintenance. The Arabidopsis CrRLK1L subfamily has 17 members, the identity and function of which have been nicely summarized in previous review articles (Lindner et al., 2012; Nissen et al., 2016; Franck et al., 2018). Here, we highlight the involvement of THESEUS1 (THE1) and FERONIA in the cell wall integrity.

As explained in the introduction, THE1 was identified from a suppressor screen in the cellulose-deficient *cesa6/prc1-1* mutant background (Hématy et al., 2007). THE1 is also required for the oxidative burst induced by the cellulose-synthesis inhibitor isoxaben in the

roots (Denness et al., 2011). Thus far, no carbohydrate components of the wall have been identified as ligands that bind THE1. Instead, a small secretory peptide, RAPID ALKALINIZATION FACTOR34 (RALF34) binds THE1 in vitro, and they may form a signaling module in vivo to maintain proper spacing among lateral root primordia (Gonneau et al., 2018). However, the molecular mechanism for exactly how RALF34 and THE1 regulate lateral root initiation, and whether they function in cell wall signaling, is unclear. One possible scenario is that THE1 is a receptor for multiple ligands and their binding is spatiotemporally dependent on developmental and environmental contexts. This possibility could also hold true for other members in the CrRLK1Ls subfamily.

FERONIA is one of the most vastly studied CrRLK1Ls. In addition, of its ability to bind pectin in vitro, FERONIA signaling works in a protein complex. FER together with glycosylphosphatidylinositol-anchored proteins LORELEI (LRE) and LRE-LIKE GPI-AP1 (LLG1), RALF and LRX was demonstrated as an alternative way of cell wall sensing mechanism. Interestingly, the triple *lrx3/lrx4/lrx5* mutant shares some phenotypic aspects of the *fer4* mutant, including stunted growth and salt stress responses, which suggests that they are active in the same pathway (Dunser et al., 2017, Zhao et al., 2018). Arabidopsis genome codes for 11 LRX genes (LRX1-11) and they are suspected to assure binding specificity towards RALF peptides in the cell wall. Pollen tubes of plants with mutations in three or more of the pollen-expressed LRX8–LRX11 were shown to be insensitive to the growth arresting effect of pollen-expressed RALF4 and showed reduced binding of RALF4 to the pollen tube surface (Mecchia et al., 2017). Crystallization of the LRX–RALF complex revealed that an LRX dimer binds two RALF peptides, exposing a highly basic surface patch of RALF that could facilitate interactions with other protein or cell wall components (Moussuet et al., 2019). The root/shoot-expressed LRX4 was subsequently found to bind RALF1, confirming that LRX proteins bind RALFs in different tissues (Dunser et al., 2019). With co-immunoprecipitation assays and yeast two-hybrid assays, a physical link between FER and the LRR domain of LRX4 was demonstrated (Dunser et al., 2019). LRX–FER-dependent cell wall sensing is required to coordinate vacuolar morphology. This process is influenced by the extracellular pH and this adjustment depends on both FER and LRX function (Dunser et al., 2019). FERONIA has also been shown to interact with LRE and LLG1 in the ER and this interaction is crucial for the localization of FER to the plasma membrane, implying that LRE and LLG1 act as chaperones for FER throughout the secretory pathway (Li et al., 2015). Thus, LRXs constitute a physical link between the plasma membrane (via the association of the LRR domain with FER) and the extracellular matrix (via the extensin domain) (Fig14).

In summary, cell integrity system was described to acts upstream of small GTPase signaling and is activated as soon as cell wall is disturbed. Therefore, we wondered if cell wall integrity mechanisms could be linked to the hyperosmotic signaling pathway. First, we explore the impact of cell wall perturbation on the osmotically induce ROS production. Then, we investigate how the sensor FERONIA is involves in the osmotic signaling pathway and finally, we test if other CrRLK1Ls can also participate to this signaling.

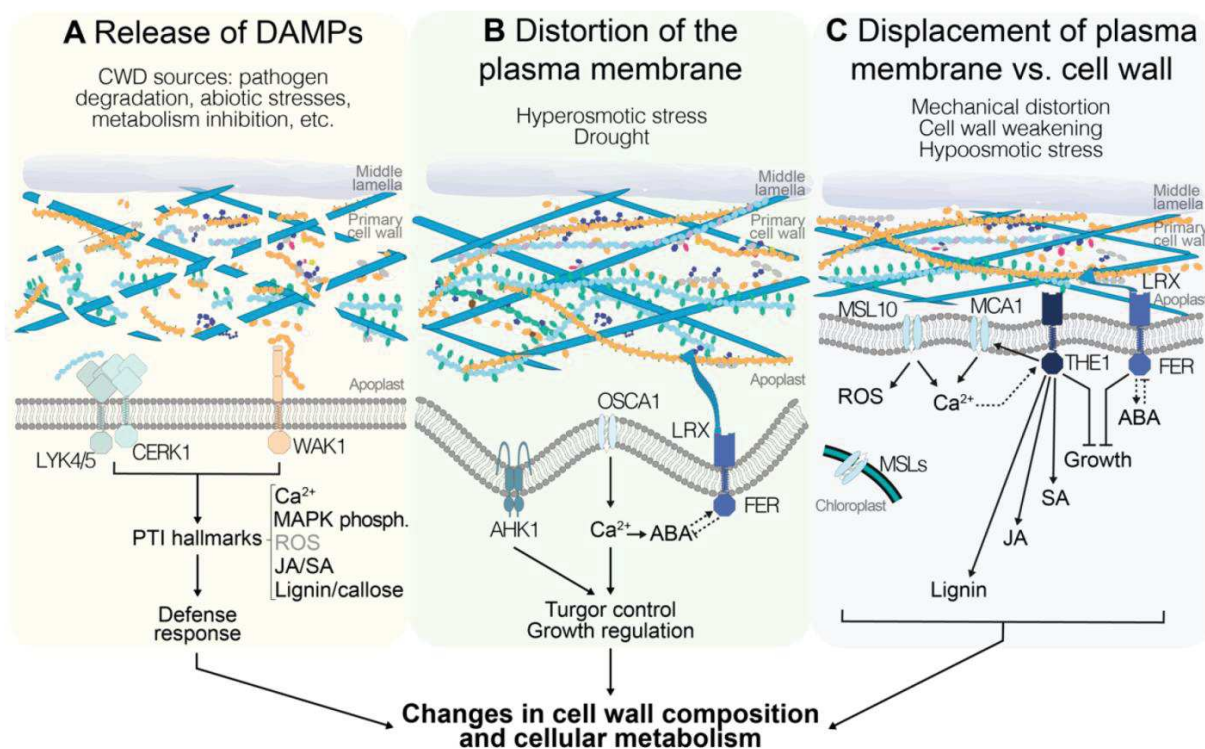


Figure 13: **Different stimuli could indicate alterations in cell wall integrity (CWI) in *Arabidopsis thaliana*, and are perceived through different signaling pathways.** (A) Release of cell wall fragments, also known as damage-associated molecular patterns (DAMPs), is interpreted by the plant as the result of cell wall damage (CWD) that can derive from biotic and abiotic stresses as well as endogenous processes. DAMP perception by pattern-recognition receptors activates typical immunity responses including increases in cytoplasmic $[Ca^{2+}]$, phosphorylation of mitogen-activated protein kinases (MAPKs), production of jasmonic acid (JA)/salicylic acid (SA), reactive oxygen species (ROS), and lignin, and callose deposition. (B) Distortion of the cell wall–plasma membrane continuum occurs in response to plasma membrane shrinkage during hyperosmotic stress (drought). Mechanosensitive ion channels mediate Ca^{2+} influx into the cytoplasm, leading to the activation of signal transduction pathways. Moreover, interactions between abscisic acid (ABA) and FER modulate growth in response to the state of turgor pressure. (C) If the plasma membrane is stretched, either by a weakened cell wall or as result of hypoosmotic stress, mechanosensitive ion channels are activated and $[Ca^{2+}]$ in the

cytoplasm is increased. Several of these channels are also required for the production of ROS. Moreover, THE1-mediated signaling modulates CWD-induced lignin and JA/SA production, and together with FER leads to growth arrest until CWI is recovered. The pathways in A, B, and C eventually lead to a series of changes in cell wall composition and cellular metabolism, enabling the plant to maintain CWI in response to different challenges. Bacete and Hamann, 2020.

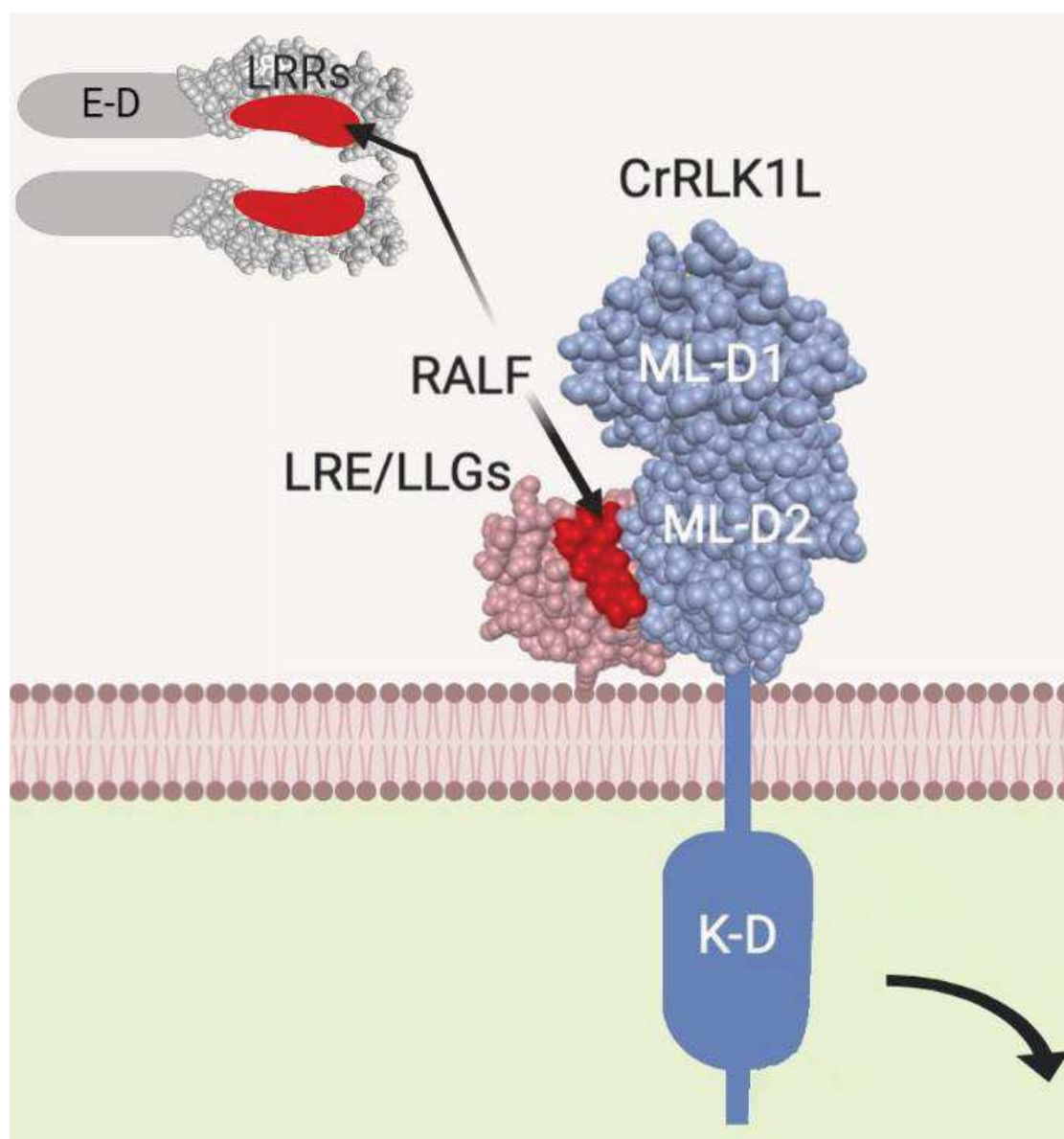


Figure 14: **Insights into RALF Perception by FER**

During RALF perception, receptor-like kinases of the CrRLK1L family interact with LRE/LLG GPI-APs in a ligand-induced manner. RALF peptides serve as molecular glue that strengthens the interaction between receptor and co-receptor, leading to intracellular signaling events. Note that the structure of the complex is restricted to the extracellular domain of the CrRLK1L kinase FER as well as to part of the co

receptor LLG2 and the N-terminal domain of RALF23. Leucine-rich repeat extension proteins (LRX) are also involved in RALF binding in the cell wall where they form a tetramer. Whether LRX proteins are linked to the membrane by replacing, for example, LRE/LLGs in the CrRLK1L receptor complexes is unclear. The hypothetical model is based on a crystal structure of RALF4 in complex with the N-terminal domain of LRR domain of LRX2. Adapted from Ge et al., 2019.

4.2 RESULTS

4.2.1 Cellulose perturbations lead to modulation of the osmotic signaling in a ROP6 dependent manner

To test the role of the cell wall during osmotic signaling, we have decided to take advantage of the isoxaben, which inhibits cellulose deposition by inducing CESA complex dissociation from the PM, and consequently blocking cellulose biosynthesis (Desprez et al., 2002; Tateno et al., 2015). ROS test based on DHE fluorescent was used as a fast landmark of osmotic signaling activation in plants. Seedlings were treated with 100nM ISX for 2h, compared to medium containing the ISX solvent dimethyl sulfoxide (DMSO). ISX treatment in Col-0 results in over accumulation of osmotically induced ROS, but no effect was seen under control situation, which would suggest that ISX treated cells are oversensitive to high osmotic environments (Fig15-A). Then, we wonder if this response could be associated to the GTPase, ROP6. The ISX effect is lost in *rop6.2* mutant and ROP6-CA that already has high ROS accumulation in control conditions is not getting higher with ISX treatment (Fig15-A). This insinuates that the ISX effect on ROS is ROP6 dependent, but that ISX is acting upstream of ROP6 activation. This hypothesis suggests that osmotic signal perception is altered in ISX treated cells. We next used mutants impaired in cellulose deposition (Fig15-B,C). Mutations in KOR totally mimic the ISX effects. *prc* and *rsw1* grown in non-permissive temperature lead to higher ROS accumulation upon osmotic stimuli, compared to Col-0. We also observed that basal ROS production is also strongly induced. *prc* and *rsw1* behave similarly as they are two mutants of the CESA genes (CESA1 and 6). But, surprisingly *rsw1* grown in permissive temperature (at 21°C) also are insensitive to hyperosmotic stress (Fig15-C). This suggests that even at this temperature the mutation in CESA6 has an effect on the cell walls. Then, we test *mur1* that have a diminished RG-II contents by ~50 (O'Neill et al., 2001) (Fig15-B). No effect on the ROS accumulation was observed suggesting that pectin might have a limited role on osmotic signaling.

To summarize, our results indicate that disruption in the cellulose structures from the cell wall leads to hypo or hypersensitivity to hyperosmotic stress. Our results suggest that cellulose perturbation acts on osmotic signaling probably upstream of ROP6.

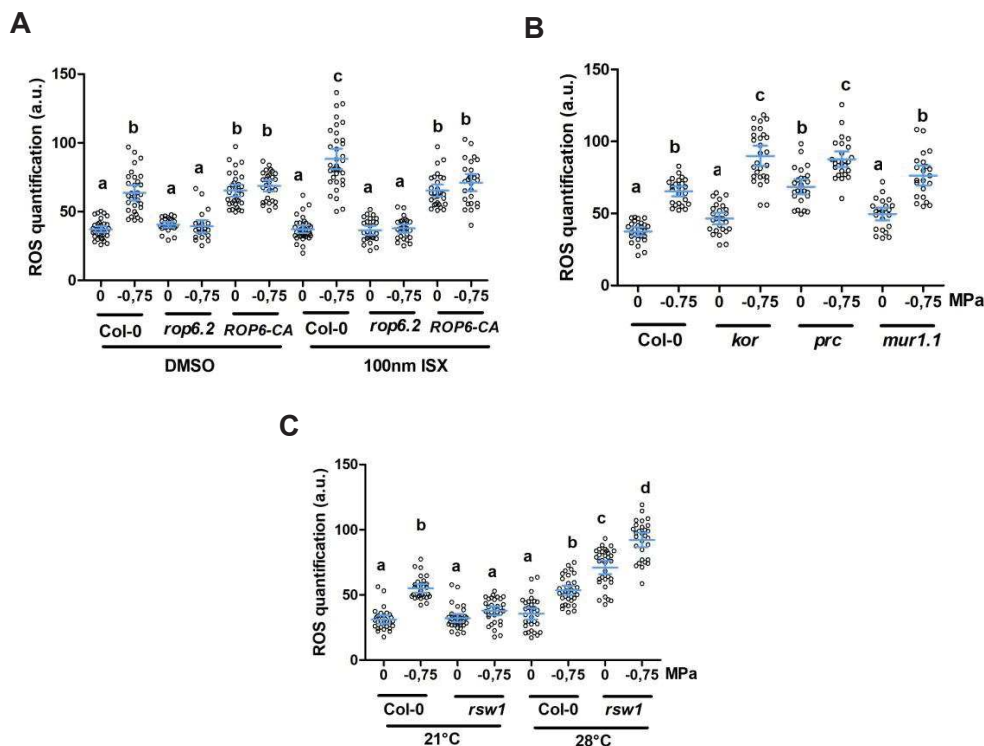


Figure 15: Disruption in cellulose biosynthesis has an effect on the osmotic stress signaling

(A) ROS quantification from DHE fluorescence signal of Col-0, *rop6.2* and *rop6.2xROP6-CA* (*ROP6-CA*) in medium containing DMSO or 100nM isoxaben incubated for 30 mins and co-treated in -0.75MPa solution for 15 mins. (B) ROS quantification from DHE fluorescence signal of Col-0 and several cellulose and pectin mutants such as *kor*, *prc* and *mur1* in control medium or incubated in -0.75MPa solution for 15 mins. (C) ROS quantification of Col-0 and the thermo-inducible *rsw1* in control or -0.75MPa solution grown at 21°C or 28°C. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p -value<0.001). $n > 20$ from 3 independent biological replica.

4.2.2 FERONIA regulates phosphatidylserine organization at the PM to modulate ROP-signaling during osmotic stress

Osmotic signal lead to profound change in the cell wall structure and can at high concentration disconnect cell wall to the PM. We have found that genetic and pharmacological perturbation of cell wall status acts on osmotic signaling. As explain in the introduction, CrRLK1Ls are PM localized kinases that transduce cell wall integrity signal to cell interior. Therefore, it is possible that CrRLK also control the osmotic signaling by activating GEF14/ROP6 module. To explore this hypothesis, we study the interaction between the cell wall sensing pathway mediated by FERONIA and the osmotic signaling pathway.

Title: FERONIA regulates phosphatidylserine nanodomains to modulate ROP-signaling during osmotic stress

(in prep)

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Abstract :

The plasma membrane and the cell wall maintained a constant dialog to ensure organism growth and development, but how this work mechanistically is still unclear. We found that the cell wall sensor FERONIA (FER) modulates the plasma membrane localization of the phospholipid, phosphatidylserin (PS) that tunes Rho GTPase signaling in Arabidopsis. With a focus on the hyperosmotic stimulus, we use loss of function approaches together with high resolution live imaging to demonstrate that FER is required for Rho of Plant 6 (ROP6) nanopartitioning at the PM that later control secondary messenger production. Our experiments also show that FER genetically acts downstream of ROP6 activation. (PS) biosensors used in combination with RALF1 and RALF 23 specific FER peptides shows that FER modulate PS nanodomain formation at the PM. Those domains been needed for ROP6 signaling in plant. Genetic and pharmacological complementations suggest that FER via PS can modulate global ROP-signaling in plants.

Introduction :

Coordination of growth and development needs a careful monitoring of environmental stimulation. Plant cells are under hydraulic turgor pressure that maintained a tight association between primary cell wall and plasma membrane. Change in cell environment water status cause an osmotic signal that lead to short and long-termed plant responses. Despite its essential role for plant abiotic stress acclimation and also cell growth and division, the perception and transducing mechanisms of the osmotic stimuli are just starting to be more elucidated (Scharwies and Dinneny, 2019,).

One of the first cellular response is an accumulation of reactive oxygen species (ROS) acting as a secondary messenger for plant responses (Leshem et al., 2007). Recently we found that ROS accumulation in cells needs the activation of the PM localized Respiratory burst oxidase homologue D and F (Rboh) (Martiniere et al., 2019). The upstream regulator has been identified as a small GTP-ase belonging to a Rho of Plants (ROP), ROP6. The osmotic signal targets within minutes ROP6 to specific nanodomains in the PM (Smokvarska et al., 2020). ROP6 nanodomains are necessary and sufficient to induce ROS in cells and also accumulate effectors protein like the Rbohs. Moreover, Platre et al 2019 described that the hyper variable tail of ROP6 was shown to interact with anionic lipid, phosphatidylserine (PS) that has a major role in ROP6 nanodomain retention upon auxin signaling. The amount of PS in plant quantitatively regulates auxin-induced ROP signaling. How ROP6 get activated upon osmotic signal remains unknown.

The molecular mechanism for osmotic signaling shared similarities with cell wall integrity pathway (CWI). This pathway transduces the cell wall status to cell interior and coordinates responses. A couple of cell wall integrity (CWI) sensors have been identified and one of them has been described to act in CWI maintenance upon environmental stresses. FERONIA (FER) belongs to the *Catharanthus roseus* (CrRLK1L) and it was described to have pleiotropic functions in a variety of cellular processes, from the preservation of cell integrity in tip-growing cells and roots elongation, to response to abiotic stress (Feng et al., 2018; Haruta et al., 2014; Duan et al., 2010; Zhao et al., 2018), Loss of FER reduced the level of activated ROPs and hampered ROP-mediated and NADPH oxidase-dependent ROS production in mutant seedlings (Duan et al., 2010; Duan et al., 2014; Li et al., 2015). The mechanism by which FER perceive cell wall status remains open. Indeed, through the two tandem malectin-like domain in its extracellular part, FER could bind pectin. But, studies show also that FER ectodomain associate to small secreted peptides the Rapid Alkalization Factor (RALFs) (Abarca et al., 2020; Haruta et al., 2014; Stegmann et al., 2017). Crystallography studies demonstrate that

RALFs can form a complex with LRR-EXTENSINS (LRXs) in the cell wall and that FER, together with the co-receptor LORELEI (LRE)-LIKE GLYCOSYLPHOSPHATIDYLINOSITOL (GPI)-ANCHORED PROTEIN (LLGs) bind also to RALFs peptides (Dünser et al., 2019, Li et al., 2015; Xiao et al., 2019, Herger et al., 2020; Zhao et al., 2018).

Under osmotic stress, the cell wall deforms and dissociates from the plasma membrane. The partial or complete loss of the interaction with the plasma membrane has a large effect on the wall and wall-associated proteins, which are anchored to the plasma membrane and are important for maintaining cell wall function. In addition, FER was shown to induce ROS production through the activation of small GTPases (Duan et al., 2010, 2014). Therefore, FER might be a good candidate for osmotic perception and downstream activation of the small GTPase ROP6.

In this study, we found that FER is surprisingly for a receptor kinase, acting downstream of ROP6 activation. Further, analysis revealed that FER regulate ROP6 nanodomain formation by controlling organization and localization at the PM of the anionic lipid, phosphatidylserine (PS).

Results

FER is a necessary component for the osmotically induced ROS accumulation

To determine if FER is involved in the osmotic stress signaling, we tested the loss of function *fer4* and a knock down mutant *fer5* (Duan et al 2010). We used treatments with low water potential solution ($\psi = -0.75$ MPa) to induce an osmotic signal to plants. ROS accumulation was used as a fast osmotic phenotypic output and it was revealed by a DHE dye (Martiniere et al., 2019) (Fig1-A). Compared to Col0, both *fer4* and *fer5* show insensitivity to the osmotic signal (Fig1-A and B). To confirm the role of CWI sensing in osmotic sensing, we tested LRX mutants that acts upstream of FER (Baumberger et al., 2001; Dünser et al., 2019; Herger et al., 2019; Herger et al., 2020; Mecchia et al., 2017). *lrx3* alone or in combination with *lrx4* or *lrx5* show impaired osmotically induce ROS accumulation (Fig1-E). Treatment with RALF1 and RALF23 peptides, two well described ligands for FER, had antagonist effects on ROS accumulation in response to osmotic stimulation. Whereas, RALF1 showed synergic ROS accumulation, RALF23 showed an inhibitory effect in osmotic co-treatment (Fig1-C,D). RALF23 is also known to have an inhibitory effect on PTI in response to bacterial elicitor flg22 via the FER pathway.(S1-A) (Stegmann et al., 2017).

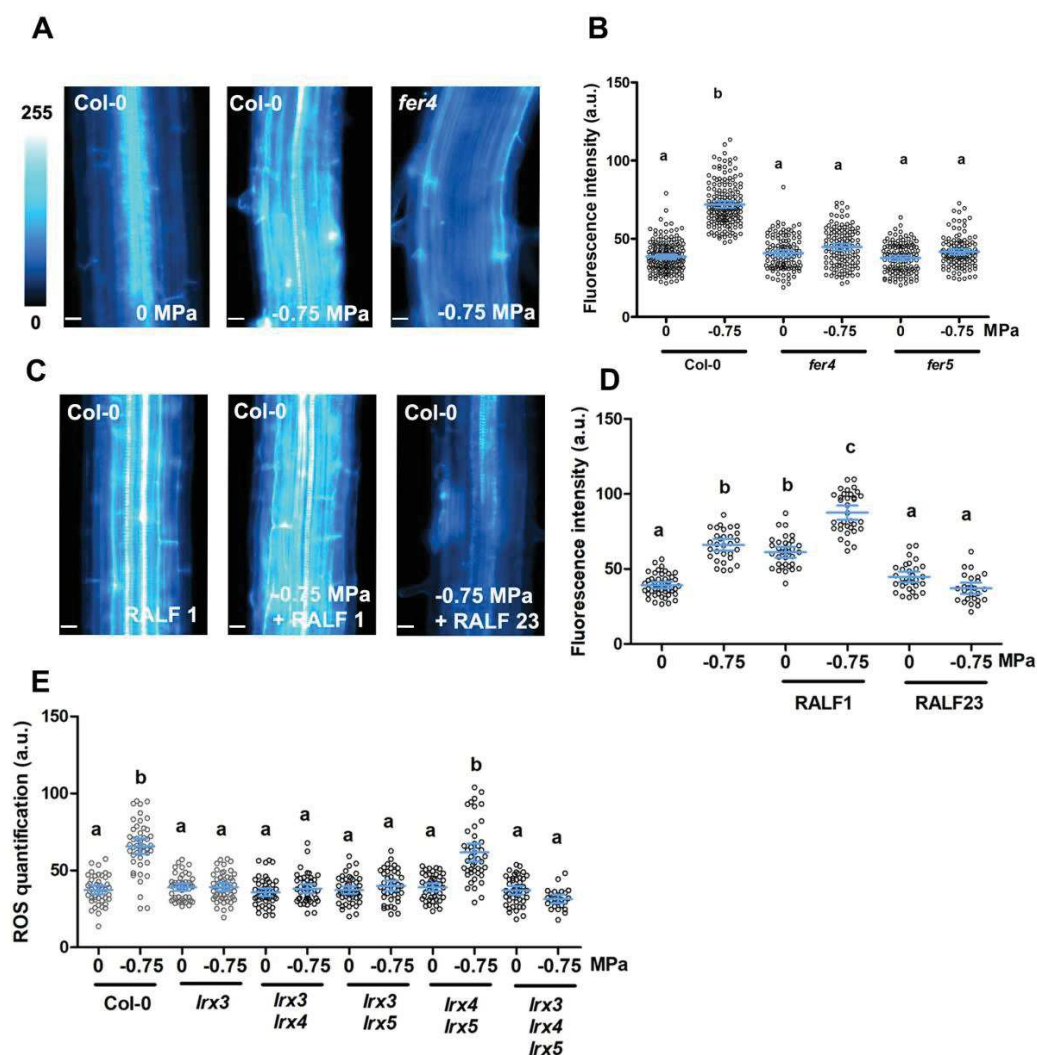


Figure 1: **FER and its co-receptor LRX3 are necessary to trigger osmotically induced ROS accumulation in plant root cells.** (A) Dihydroethidium (DHE) stained root cell of Col-0 or *fer4* in control condition (0 MPa) or after 15 min of -0.75 MPa treatment (B) DHE fluorescence quantification after 15 min treatment with 0, or -0.75 MPa solution in different genetic material: Col-0, *fer4* (KO) and *fer5* (KD). (C) Dihydroethidium (DHE) stained root cell of Col-0 pretreated with 1 μ M RALF1 or RALF23 for an hour followed by control condition (0 MPa) or -0.75 MPa treatment for 15 min and its quantification (D). (E) ROS quantification from DHE fluorescence signal of several genotypes: Col-0, *lrx3*, double *lrx3/lrx4*, *lrx3/lrx5*, *lrx4/lrx5*, and triple *lrx3/lrx4/lrx5* mutant in control or -0.75MPa treatment. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p -value<0.01). n >26 from 3-5 independent biological replica. Scale bar 10 μ m.

FERONIA acts downstream of ROP6 and has no effect on its protein quantity nor localization

As FER is a receptor kinase, we hypothesized that it might act upstream of the small GTPase ROP6. To test this hypothesis, we took advantage of plant expressing GTP-locked ROP6 (mCit-ROP6gCA) which has constantly high ROS accumulation irrelevant from the condition (Smokvarska et al., 2020). Both knock down and knock out alleles of FER inhibit the mCit-ROP6gCA phenotype (Fig2-A). Furthermore, treatment with RALF23 peptide inhibited the constitutive ROS production of the mCit-ROP6gCA line (Fig2-B). These results suggest that FER acts downstream of the constitutively activated ROP6, and confirm that RALF23 negatively regulates this pathway.

We next, wondered if the *fer4xROP6-CA* phenotype might be caused by reduced amount of mCit-ROP6gCA. Indeed, FER was described to modulate both gene splicing and protein translation (Zhu et al., 2020) (Wang et al., 2020). No difference in mCit-ROP6gCA quantity was observed between Col0 and *fer4* or *fer5* (Fig2-C). Next we check if mCit-ROP6g-CA could be mislocalized in FER mutant backgrounds. Although, the mCit-ROP6g-CA intensity is higher in *fer5* than in *fer4* or WT; the ratio between PM and intracellular labeling is the same between all genotypes (Fig2-D, E, F). Consequently, protein quantity or plasma membrane targeting cannot explain the inhibitory effect of the FER mutation over GTP-lock ROP6 phenotype.

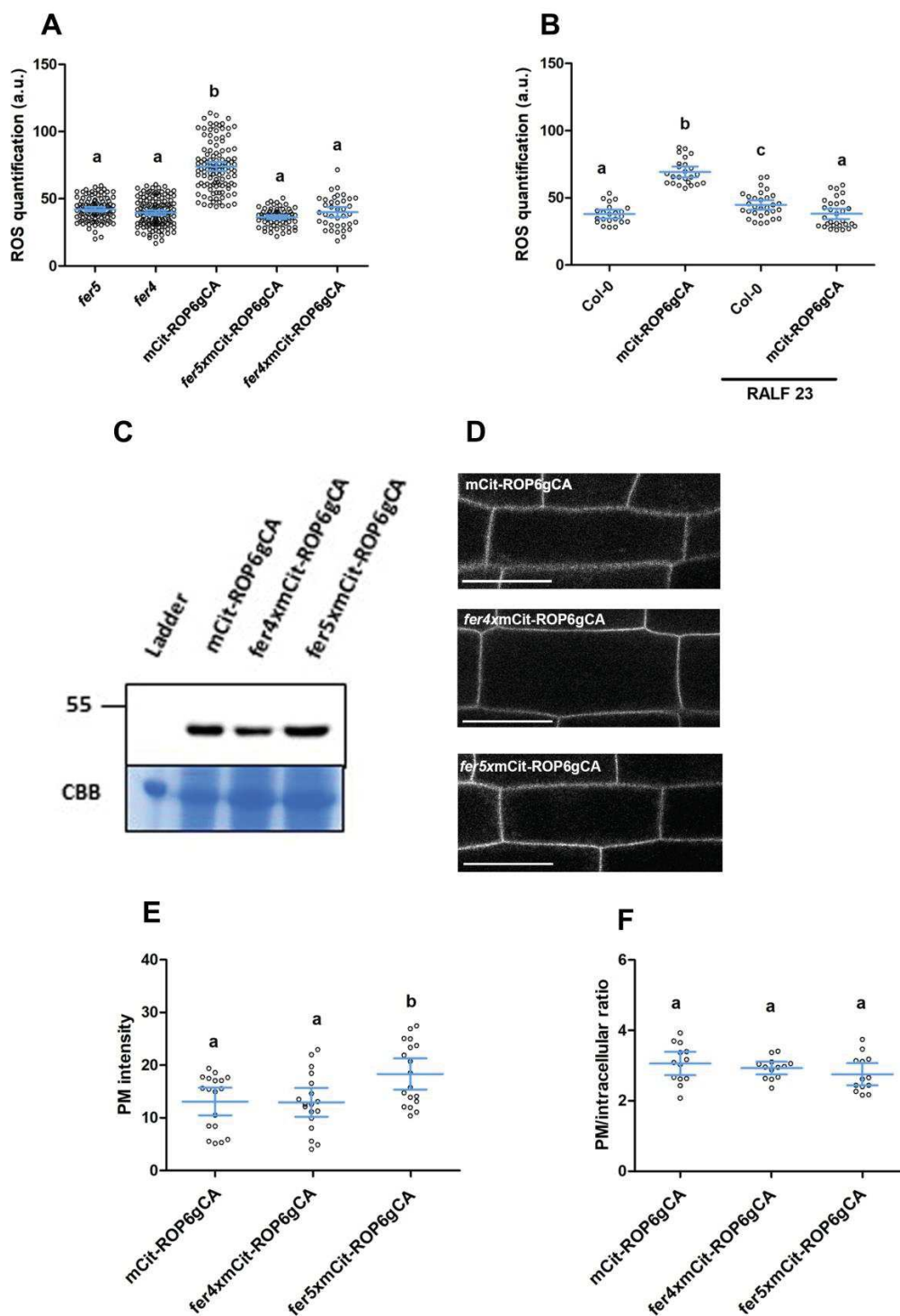


Figure 2: FER acts downstream of activated ROP6 and do not affect mCit-ROP6g-CA protein quantity or localization. (A) ROS quantification from DHE fluorescence signal of several genotypes: *fer4*, *fer5*, mCit-ROP6gCA, *fer4xmCit-ROP6gCA* and *fer5xmCit-ROP6gCA*. (B) ROS quantification from DHE fluorescence signal of 1µM RALF23 peptide for 1 hour on Col-0 and mCit-ROP6gCA. (C) Western

blot with antibody against GFP on plant protein extract from mCit-ROP6gCA, *fer5xmCit-ROP6gCA* and *fer4xmCit-ROP6gCA*. (D) Confocal micrograph showing the localization of mCit-ROP6gCA, *fer5xmCit-ROP6gCA* and *fer4xmCit-ROP6gCA*. Quantification of the mCitrin signal at the PM (E) and of the PM/intracellular mCitrin signal ratio of mCit-ROP6gCA, *fer5xmCit-ROP6gCA* and *fer4xmCit-ROP6gCA* (F). Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p-value<0.001). n>10 from at least 2 biological replicates. Scale bar 20 μ m.

FER regulates the formation of PS-containing nanodomains that tune ROP6 recruitment in response to osmotic stimuli

Because we did not observe an obvious phenotype of a miss localization of ROP6 from the PM in loss of function FER (Fig2-D,E,F), we hypothesized that FER might regulates the spatial organization of ROP6 within the membrane. ROP6 is targeted to PS-enriched nanodomains when it gets activated (Platre et al 2019). To test a potential link between ROP6-containing nanodomains, PS and FER, we first tested the role of PS during osmotic signaling. *pss1-3*, that do not have PS, show no osmotically induce ROS accumulation (Fig3--A; Platre et al., 2018). PSS1-OX line, that have two third more PS than control plants, did not reveal any further enhancement of ROS production, suggesting that PS is not a limiting factor during osmotic signaling (Fig3-A; Platre et al., 2019).

We, next, wondered if the FER loss-of-function phenotype (Fig1-B) is due to low PS quantities. Lipid quantification by high performance thin layer chromatography (HPTLC) analyses showed that *fer5* allele has more PS than Col0 and that *fer4* allele present an excess of PC and PE, but similar PS compare to wild type (Fig3-B). Thus, even if those differences are significant they show some discrepancy between the two alleles and consequently PS quantity in FER mutants do not clearly explain their signaling defaults. Because FER was described as a scaffold PM kinase, we examined if disruption in FER changes PS localization in the membrane (Stegmann et al., 2017). We used two well described PS biosensors, 2xPH^{EVECTIN} and C2^{LACT} (Platre et al., 2018, Simon et al., 2016) and introgressed them in *fer4* or *fer5* mutant background. We observed a depletion of PM signal from both sensors in the two mutant alleles of FER. (Fig3-,C,D,E). This effect was not observable in *rop6.2* confirming that ROP6 do not control PS localization at the PM (S2-A,B). Then, we used RALF1 and RALF23 exogenous application to activate or inhibit FER pathway. Whereas RALF1 induced PS sensor localization at the PM, RALF23 reduced it. (Fig 3-C,F,G). This effect was totally abolished in *fer4* background, demonstrating that functional FER is needed to relocalize PS after RALF peptide treatments (S2-C, D). By contrast to the FER/RALF pathways, osmotic signal had no impact on the localization of mCIT-C2^{LACT} (S2-E,F).

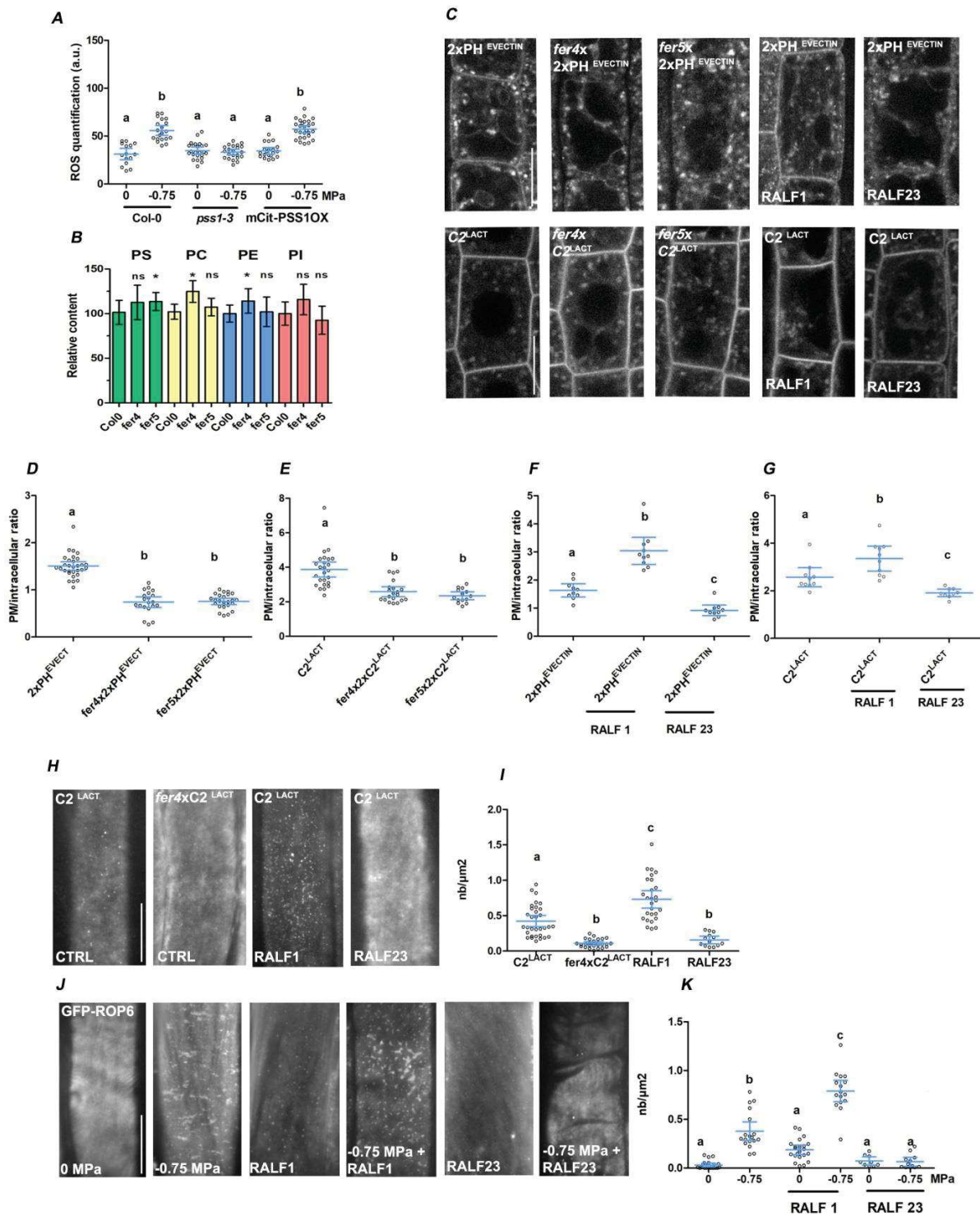


Figure 3: PSS1 is needed for proper osmotic signaling , FER regulate phosphatidylserine localization and this in return modulates ROP6 dynamics.(A) ROS quantification from DHE fluorescence signal of Col-0, *pss1-3* mutant, lacking PS and PSS1 overexpressed line in control or hyperosmotic treatment (-0.75MPa). (B) Quantification of the PS, PC, PE and PI by HPTLC in Col-0, *fer4* and *fer5*. *fer4* and *fer5* values are shown in relative content compared to Col-0. (C) Confocal micrograph showing the localization of the two PS biosensors 2XPH^{EVECTIN} and C2^{LACT} in WT and *fer4* or *fer5* mutant background; RALF1 and RALF23 peptides effect on the two PS sensors. Quantification of the PM/intracellular m-citrine signal ratio of 2XPH^{EVECTIN}, *fer4*x2XPH^{EVECTIN}, *fer5*x2XPH^{EVECTIN} (D); C2^{LACT}, *fer4*xC2^{LACT}, *fer5*xC2^{LACT} (E); RALF1 and RALF23 effect on 2XPH^{EVECTIN} (F) and RALF1 and RALF23 effect on C2^{LACT} (G). (H) TIRFM micrograph of C2^{LACT}, and *fer4*xC2^{LACT} in control condition or under RALF1 and RALF23 treatment with the corresponding cluster density quantification (I). (J) TIRFM micrograph of GFP-ROP6 overexpressed line in control, -0.75 MPa solution or combined treatment with RALF1 or RALF23 peptides and quantification of GFP-ROP6 cluster density (K). Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p-value<0.001). In (B) paired Wilcoxon test was done comparing *fer4* to Col-0 and *fer5* to Col-0 for each lipids separately For (B) n>7, (A,C,D,E,F,H,J,K) n>14 from at least 2 independent biological replicates. Scale bar 20µm (B) 10µm (H,J).

Our results suggest that FER pathway control PS quantity and/or availability at the PM. We thus wondered if it could also control its nanodomain distribution. We applied total internal reflection (TIRF) microscopy on Col0 or *fer4* expressing C2^{LACT}. The dotted structures observed in wild type plants were totally absent in FER loss-of-function background (Fig3-H,I). Interestingly, RALF1 treatment shows a significant enhancement of C2^{LACT}-containing nanodomains and oppositely RALF23 treatment showed no nanodomains, similar to the *fer4xC2^{LACT}* phenotype (Fig3-H,I). Then, we hypothesized that FER pathway might prime ROP6 recruitment into PS nanodomains. To test this, we looked at GFP-ROP6 localization under TIRF to record ROP6 recruitment in nanodomain upon osmotic signal. As expected, osmotic signal induced the recruitment of GFP-ROP6 into nanodomains. The density of nanodomains that contain GFP-ROP6 was higher if cell were pretreated with RALF1 and reduced up to the non-stimulated control if RALF23 was present (Fig3-J,K). These data overlaps with the additive and inhibitory effect on RALF1 and RALF23, respectively, on the ROS accumulation (Fig1-D), showing that FER regulates the formation of PS-containing nanodomains that later acts on ROP6 recruitment.

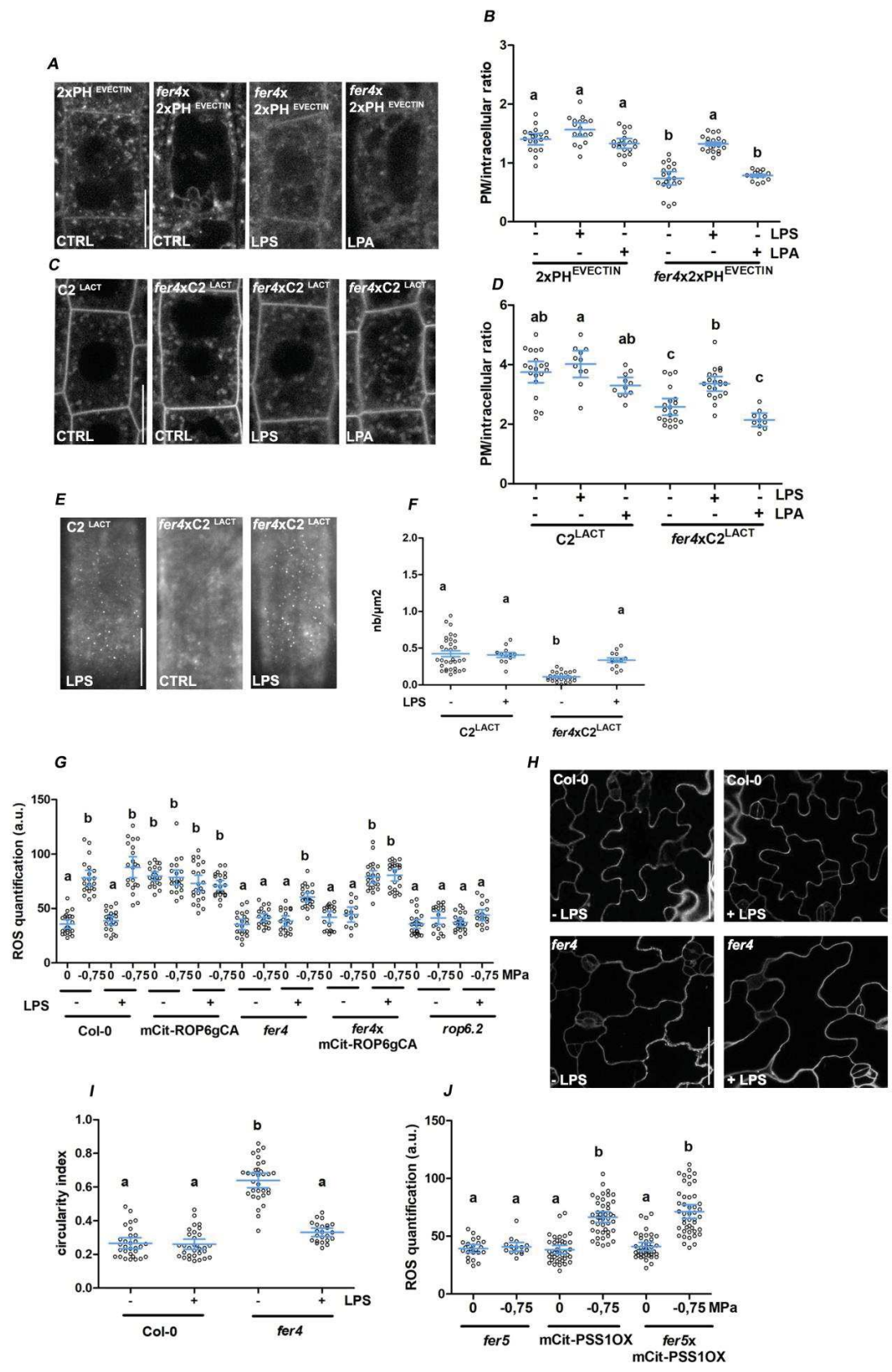


Figure 4: **Genetic overexpression or exogenous addition of PS, but not PA can reverse *fer*-mutation related phenotypes.** (A) Confocal micrograph showing the localization of *fer4x2XPH^{EVECTIN}* with or without LPS and LPA media supplementation and the corresponding quantification of the PM/intracellular ratio of m-Citrine signal of respected constructs (B). (C) Confocal micrograph showing the localization of *fer4xC2^{LACT}* with or without LPS and LPA and its quantification (D). (E) TIRFM micrograph of *fer4xC2^{LACT}* in an incubation media supplemented or not with LPS with cluster density quantification (F). (G) ROS quantification from DHE fluorescence signal of several genotypes: Col-0, ROP6-CA, *fer4*, *fer4xROP6-CA*, *rop6.2* Col-0 in control condition (0 MPa) or after 15 min of -0.75 MPa treatment with (+) or without (-) 1h LPS pretreatment. (H,I) Confocal micrographs of cotyledon pavement cells (PC) revealed with propidium iodide of Col-0 and *fer4* and quantification of the PC circularity index measurement. Close the values are to 1, more circular the cells are.(J) ROS quantification from DHE fluorescence signal of PSS1 overexpression line in either WT or *fer5* background.

Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p-value<0.001). For (A) n>7, (C,D,E,F,H,J) n>12 from at least 2 independent biological replicates. Scale bar 20µm (A,C,H), 10µm (E)

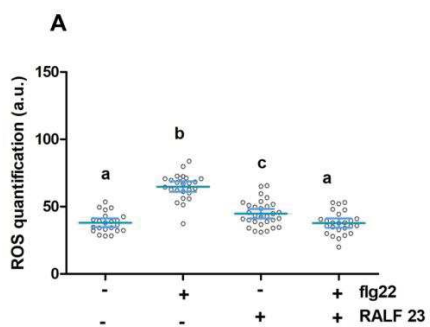
PS nanodomains acts downstream of FER.

To determine if PS nanodomain formation is regulated by the FER pathway, we used exogenous application of lysophospholipids. They are more soluble than phospholipids and as such are more likely to reach the cytosolic leaflet of cellular membranes (Maeda et al., 2013, Moser von Filseck et al., 2015). This approach has already been proven functional where short-term (i.e. 1 hour) treatment with Lyso-PS (LPS) can rescue the membrane localization of a PS sensor in the *pss1* mutant and long-term treatment can partially reverse *pss1-3* root developmental phenotype (Platre et al dev cell). Root of *fer4x2xPH^{EVECTIN}* and *fer4xC2^{LACT}* supplemented for 1 hour with LPS (Fig4-A,B,C,D) showed a relocalization of PS biosensors back to the PM, making them similar to wild type plants. Similar results were found with the knock down allele *fer5* (S3-A,B,C,). Plate supplemented with Lyso-PA (LPA) did not allowed relocalization of both PS biosensors, even it was functional in our experiment as it can complement PM localization of the PA biosensor (2xPASS) after 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) treatment that inhibit PLD activity (Fig4-B, D) (S3-B, C, D). If LPS application reorients PS to the PM in FER loss-of-function, could it also complement PS organization at the PM? TIRF observation revealed that LPS can also complement the C2^{LACT} clustering that is missregulated in *fer4* mutant (Fig4-E,F).

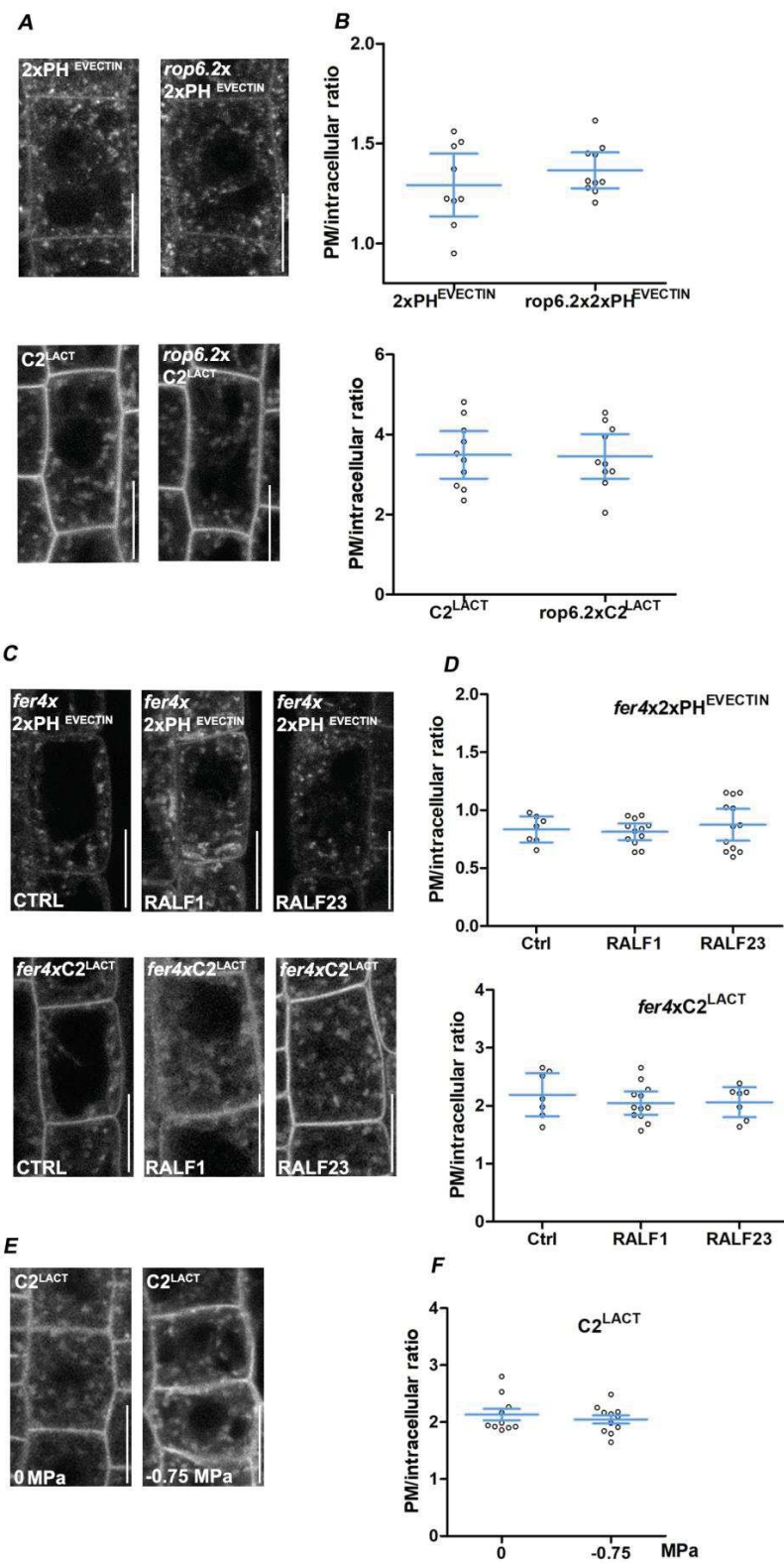
Since LPS can complement C2^{LACT} and 2xPH^{EVECTIN} localization in *fer* mutants, we wondered if it could rescue osmotic signaling pathway related to ROS. ROS accumulation in roots was quantified in Col0, *fer4* and *fer5* after osmotic stimulation in presence or absence of LPS in the media. Exogenous addition of LPS can rescue the osmotically induced ROS of *fer4* (Fig4-G) and *fer5* (S3-E). LPA on the other hand has no effect on the *fer4* and *fer5* ROS response (S3-F). To confirm the role of PS in osmotically mediated ROS accumulation, we used plants overexpressing PSS1 which has higher quantity of PS. *fer5xmCit-PSS1ox* line show similar behavior to *fer5* treated with exogenous LPS (Fig4-J). This suggests that pharmacological or genetic modulation of PS can both overcome the loss of FER function in plants. Interestingly, the localization at the ER of mCit-PSS1ox is not modified by the absence of functional FER protein, suggesting that FER do not control the localization of PSS1 (S3-G).

Then, we wondered if LPS treatment can acts on ROP6 activation. To do so, we compared ROS accumulation in mCit-ROP6gCA and *fer4xmCit-ROP6gCA* lines. Exogenous LPS reverts the absence of ROS in *fer4xmCit-ROP6gCA* independently of the osmotic signal (Fig4-G). Oppositely, the loss of osmotically induced ROS accumulation present in *rop6.2* cannot be reverted by LPS treatment (Fig4-G). These data point that PS act upstream of ROP6 activation.

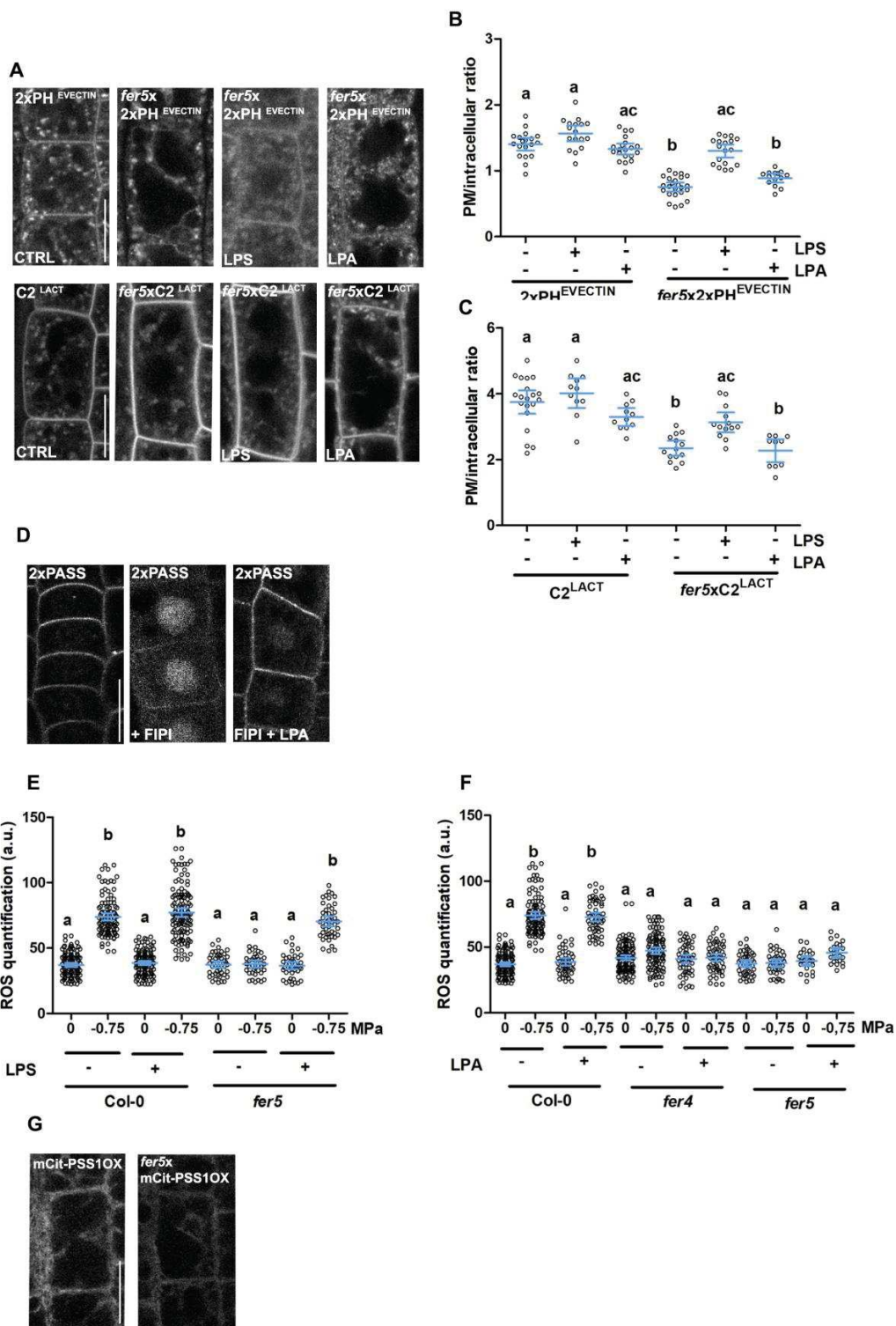
All these data show that FER can control protein clustering at the PM by acting on lipid organization. Then, we tested if this could also control other cell signaling pathway than the triggered by osmotic treatment. FER pathway is known to control PAMPs triggered immunity (PTI) in plant by regulating protein scaffolding at the PM (Stegmann et al., 2017). It regulates the complex formation between FLS2 and BAK1 that is needed for ROS accumulation in cells upon flg22 bacterial elicitor treatment (Stegmann et al. 2017). As a consequence, FER loss-of-function plants present default ROS after flg22 and we tested if exogenous LPS treatment can revert this phenotypes. LPS, but not LPA complement flg22 dependent ROS accumulation in roots (S4-A, B). This result suggests that PS localization regulated by FER can acts onto other signals. FER and ROPs was also associated to cell polarity (Pan et al., 2020, Duan et al., 2010). FER loss of mutant, but also multiple mutants of ROPs were reported to have disrupted pavement cell shape, resulting in large circular cells (Fu et al., 2005). Interestingly, similar phenotype was described for *pss1-3* (Platre et al 2019 science). By growing *fer4* plantlets for 3 days on media containing LPS, we observed restoration of normal PC cell shapes with indentations and lobes (Fig4-H,I). This rescue of PC shape is not visible upon LPA treatment in *fer4* (S4-C). Together, this set of results suggests that regulation of PS localization by FER is a determinant for several plant signaling and development pathways.



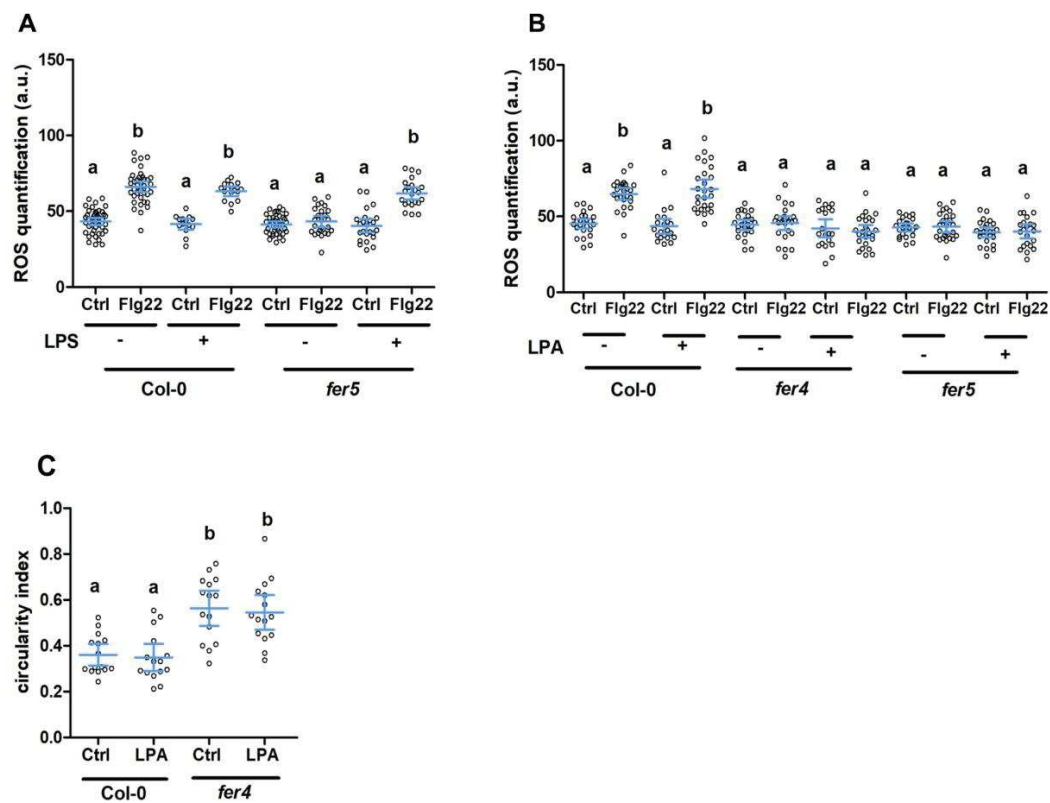
Supplemental figure 1: **RALF23 can inhibit the flg22 produced ROS** (A) ROS quantification from DHE fluorescence signal of Col-0 treated for either 30 min with 1 μ M flg22, 1h 1 μ M RALF23 pretreatment or combined RALF23 and flg22 treatment on root epidermal cells. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p-value<0.001). n>20 from 3 independent biological replicates.



Supplemental figure 2: **PS biosensors are not affected by RALF1 and RALF23 in *fer* background and hyperosmotic stress nor ROP6 mutation affect the PS localization.** (A) Confocal micrographs of $2xPH^{EVECTIN}$, $rop6.2x2xPH^{EVECTIN}$, $C2^{LACT}$ and $rop6.2xC2^{LACT}$ with their proper quantification (B). (C) Confocal micrographs of $fer4x2xPH^{EVECTIN}$ and $fer4xC2^{LACT}$ with RALF1 and RALF23 treatment and their corresponding PM/intracellular ratio quantification (D). (E) Confocal micrographs of $C2^{LACT}$ in control or -0.75MPa treatment with corresponding quantification (E). ANOVA followed by Tukey test and t-test indicate values differences are non significant. $n>10$ from at least 2 independent biological replicates (except in E,F where data is from one biological relipate). Scale bar 20 μ M.



Supplemental figure 3: **Exogenous overloading the cells with PS and not PA can complement PS miss regulation in *fer5* mutant and osmotically induced ROS accumulation.** (A) Confocal micrographs of *fer5x2xPH^{EVECTIN}* and *fer5xC2^{LACT}* with or without LPS and LPA supplementation. (B,C) Quantification of the PM/intracellular ratio, of the both PS sensors in *fer5* background, with LPS or LPA supplementation. (D) Confocal images of PA sensor 2xPASS localization with PA inhibitor, FIPI, show that LPA can complement the 2XPASS sensor localization. (E) ROS quantification from DHE fluorescence signal of Col-0 and *fer5* in control or 15 min -0.75MPa treatment with (+) or without (-) LPS supplementation. (F) ROS quantification from DHE fluorescence signal of Col-0, *fer5*, and *fer4* in control or 15 min -0.75 MPa treatment with (+) or without (-) LPA supplementation. (G) Confocal micrographs of ER labeled PSS1 OX in WT or *fer5* background. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p -value <0.001). $n>12$ from at least 2 independent biological replicates. Scale bar 20 μ m.



Supplemental figure 4: **flg22 ROS induction in fer mutants can be complemented by LPS but not LPA.** (A) ROS quantification from DHE fluorescence signal of Col-0 and *fer5* in control or after 30 min flg22 treatment, pre-treated with or without LPS. (B) ROS quantification from DHE fluorescence signal of Col-0, *fer4* and *fer5* in control or after 30 min flg22 treatment, pre-treated with or without LPA. (C) circularity index quantification of Col-0 and *fer4* PC supplemented or not with LPA. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p -value <0.001). $n>12$ from at least 2 independent biological replicates.

Discussion

In the course of these years FER have been extensively studied and been described to participate in many physiological aspects (Franck et al., 2018,). In fact, FER has been shown to control in auxin-induced root hair growth, ABA-induced guard cell turgor regulation (Yu et al., 2012), and pathogen response in the leaf (Kessler et al., 2010; Stegmann et al., 2017). In this last example, the authors have shown that FER is acting as scaffolding protein to maintained/provoke the complex between the flagellin receptor FLS2 and the co receptor BAK1, mediating the downstream plant response to PAMPs elicitors (Stegmann et al., and Gronnier et al., 2020 BioRxiv). Our results show that FER pathway act as modulator of the strength of the osmotic mediated ROS accumulation. This mechanism appears to be regulated by the number of PS nanodomains at the PM, which is latter a platform for ROP6 recruitment. Like for PAMPs signaling, FER appear as an indirect modulator of the signaling since the quantity of PS at the PM is not regulated by the osmotic signal.

FER seems to be a regulator of PS nanodomain density at the PM. Previous work on ROP6 and auxin signaling has clearly established that ROP6 is interacting with PS through its hyper variable tail (Platre et al., 2019). The PS depletion at PM, like in *PSS1* loss of function induces a weaker retention of ROP6 in nanodomains. This loss of ROP6 retention in nanodomains was associated to a loss of ROP6 signaling in the *pss1.3* mutants. Whether PS nanodomain density and PS quantity within nanodomains are regulated by similar processes, are at the moment unexplored and could be two ways to modulate spatially ROP signaling. The role of PS for ROP signaling might be extendable to other ROP isoform. Indeed, ROP6 has only a limited role on the pavement cell (PVC) shape, whereas this process is altered both in multiple ROPs mutant or overexpression of dominant negative or autoactive ROP. Our ability to complement the PVC developmental phenotype of FER loss of function, suggest that PS nanodomain is likely needed for other ROP signaling processes that are mediating by other type of ROP effectors, e.g. RIC1/Katanin (Pan et al. 2020.) The pleiotropic effect of FER on cell signaling is also supported by the fact that exogenous application of LPS can also complement *flg22* mediated ROS accumulation in roots. This suppose that FER scaffolding feature need a specific PS organization at the PM, maybe acting on the maintenance of the localization of RAFL/FER/LGG complex.

The FER pathway modulates the PS organization at the PM, but without strong changes in terms of PS quantity in the plant. Similarly, we found that both RALF1 and RALF23 peptides and FER loss of function have altered PS localization. Those effects were complemented by exogenous application of LPS but not LPA. This result suggests that FER is acting on PS

localization at the PM. How this could work mechanistically is currently unknown, as we do not know how PS gets to the PM after being produced in the lumen of the ER. Cell trafficking by exocytosis/endocytosis is unlikely since, RALF1 peptide treatment was shown to induce endocytosis of many PM proteins, and therefore is not fitting with our RALF1 induce PS nanodomain formation (Xiao et al., 2002). In yeast and mammalian cells the PS targeting is mediate at least for a part through ER/PM contact site (Scorrano et al., 2017). Certain isoforms of OSP protein transfer PS from the ER to the PM (Antonny et al., 2018). Among other, this could be a target of FER activity to regulate PS nanodomain at the PM.

As previously described, FER activation is happening in the cell apoplasm. Especially, RALF perception by FER involves additional proteins that bind the cell wall, such as the LRX (Dünser et al., 2019; Herger et al., 2020; Zhao et al., 2018). LRXs constitute a physical link between the plasma membrane (via the association of the LRR domain with FER) and the cell wall (via the extensin domain). A membrane association of LRXs lacking the extensin domain supports this hypothesis (Fabrice et al., 2018). Where LRX3,4,5 are needed for salt-induced FER endocytosis, we showed that LRX3 alone seems necessary for proper osmotic signaling. Interestingly, LRX3 was shown to be able to bind both RALF1 and RALF23 (Dunser et al. 2019, Zhao et al. 2018). The LRX/RALF binding might be regulated by the pH and redox potential of the cell wall, allowing transient dissociation of the RALFs peptides that could be later perceived by FER. Therefore, several examples in the literature suggest that FER response is linked to cell wall status perception. Then, as FER regulates PS nanodomain, it's possible that the cell wall status can be translated in the PM by a modulation of its organization/composition. Thus, this information can be used to modulate cell signaling events like the ROP GTPase signaling.

Material and methods

Plant material and growth

Arabidopsis thaliana ecotype Colombia (Col-0) was used as WT control. Following lines were previously published: *fer4*, *fer5* (Duan et al., 2010, Chen et al., 2016), *rop6.2xmCit-ROP6gCA*, *p35S:GFP-ROP6* (Smokvarska et al.2020), *UBQ10prom::mCitrine-2xPASS*(NASC# N2107781), *pss1-3* (Platre et al., 2018); *UBQ10prom::mCitrine-2xPH^{EVCTIN}*(NASC# N2107779), *UBQ10prom::mCitrine-C2^{LACT}* (NASC#N2107347), *PSS1-OX(UBQ10prom::PSS1-mCitrine)* (Platre et al 2019), *lrx3*, *lrx3/4*, *lrx3/5*, *lrx4/5*, *lrx3/4/5* (Dunser et al., 2019). Crosses *fer4xpromROP6:mCitrine-ROP6-CA*, *fer5xpromROP6:mCitrine-ROP6-CA*, *fer4x2xPH^{EVCTIN}*, *fer5x2xPH^{EVCTIN}*, *fer4xC2^{LACT}*, *fer5xC2^{LACT}*, *fer5xPSS1-OX* were done in this study. Plants were stratified for 2 days at 4°C and grown vertically on agar plates containing half-strength Murashige and Skoog (½ MS) medium supplemented with 1% (w/v) sucrose and 2.5mM MES-KOH pH6 for 5 days at 22°C in a 16-h light/8-h dark cycle with 70% relative humidity and a light intensity of 200µmol·m⁻²·s⁻¹.

ROS quantification

The ROS quantification assay is as described in Martiniere et al 2019 and Smokvarska et al 2020. In brief, to quantify the ROS production, 5µM dihydroethidium (DHE) dye was used. 5 days old plantlets were incubated in MS/2 liquid media to rest for 30 min, then transferred into 300 mM sorbitol (-0.75 MPa) for severe osmotic stress for 15 minutes, RALF1 and RALF 23 (1 µM, 1h), Flagelin 22 (1µM,30 mins). Observations were performed on the transitional zone between the elongation and differentiation zone with inverted fluorescent microscope Zeiss, 20X objective, using wavelength filter of 512/25 nm of the microscope lumencor with emission filter of 600/50 nm. The exposure time was 500 ms. Images were acquired using CDD camera (Cooled SNAP HQ, Photometric), controlled by the fluorescence Ratio Imaging Software (Metafluor).

Subcellular and Phenotype Complementation with Lysophospholipids

For complementation of the osmotically or flg22 induced ROS *fer4* and *fer5* were treated with Lyso-PS (LPS) or LysoPA (54µM, 1h) prior to imaging. For complementation of the subcellular localization PS sensors *mCitrine-2xPH^{EVCTIN}* and *C2^{LACT}* introgressed or not in *fer4* or *fer5* were treated with LPS or LPA (54µM, 1h). PA sensor *mCITRINE-2xPASS* were concomitantly treated with 1µM FIPI and lysophosphatidic acid (LPA, 54mM, 1h). For complementation of the pavement cells plantlets from Col-0, *fer4* and *fer5* were grown on regular ½ MS media for 3 days then transferred to media containing or not LPS or LPA (2.2µM) for additional 3 more days. Pavement cells were revealed with 1mg/ml propidium iodide stained for 20 mins and

then washed in PBS for 5 mins. Circularity index was measured with ImageJ tool: shape description.

Western blot

Tissues from 5 days old mCitrine-ROP6-CA, *fer4xmCitrine-ROP-CA* and *fer5xmCitrine-ROP6-CA* grinded with liquid nitrogen and resuspended in 1 mL/g powder of RIPA extraction buffer (150 mM NaCl, 50mM Tris-HCl, pH- 8, 0.1% SDS, 0.5% Na deoxycholate, 1% Triton x-100, 2mM leupeptin, 1mM PMSF and 5mM DTT). α -GFP-HRP antibody for western blot was diluted in blocking solution (1% BSA in 0.1% Tween-20 and PBS) at 1:2000. Total protein quantity was revealed with Commasie blue stain.

Confocal laser scanning microscopy

Signal from the mCitrine-ROP6-CA, *fer4xmCitrine-ROP-CA*, *fer5xmCitrine-ROP6-CA*, mCitrine-2xPH^{EVCTIN}, mCitrine-C2^{LACT}, *fer4x2xPH^{EVCTIN}*, *fer5x2xPH^{EVCTIN}*, *fer4xC2^{LACT}*, *fer5xC2^{LACT}*, *fer5xPSS1-OX* were imaged with Leica SP8 microscope with a 40 \times /1.1 water objective. 514 nm of the Argon laser was used and fluorescence emission was collected at 530-560nm for mCitrine tagged lines. To quantify the plasma membrane/intracellular ratio on phosphatidylserine biosensors and mCitROPgCA, we calculated and analyzed the “Ratio plasma membrane/intracellular fluorescence intensity”. This correspond to the ratio between the fluorescence intensity (Mean Grey Value function of Fiji software) measured along the plasma membrane region and four elliptical ROIs inside the cell (cytosol and intracellular compartments).

TIRF microscopy

For cluster density analysis of mCitrine-C2^{LACT}, *fer4xmCitrine-C2^{LACT}* and GFP-ROP6, in either resting conditions or treated with sorbitol, RALF1, RALF23 or LPS, total Internal Reflection Fluorescence (TIRF) Microscopy was done using the inverted Zeiss microscope and a 100 \times /1.45 oil immersion. 100 images were acquired with 50ms exposure time at 50 gain, with 475 nm excitation and 530/25 nm emission. Images were Z stacked by average intensity. Image segmentation for nanodomain density calculation was done using machine-learning based software iLastik (Berg et al., 2019).

Lipid extraction and lipid quantification

5 days old Col-0, *fer4* and *fer5* plants (0.1-1g fresh weight) were collected in glass tubes; 2 ml of preheated isopropanol were added and tubes were heated at 70°C for 20 min to inhibit phospholipase D activity. 6 ml of chloroform/methanol 2/1 (v/v) were added and lipid extraction was completed at room temperature. The organic phases were transferred to new glass tubes. Then 1.5 ml of H₂O was added to the organic phases and tubes were vortexed and

centrifuged at 2000rpm; the organic phases were transferred to new glass tubes, evaporated and the lipids were resuspended in the appropriate volume of 11chloroform/methanol 2/1, v/v, in order to obtain the same concentration according to the initial seedlings fresh weight. Lipid quantification was performed as described by Platre et al., 2018.

Statistical analysis

In each experiment, 8 to 10 cells are studied from 5 to 7 different seedlings. All experiments were independently repeated 2-3 times. Data are expressed as mean \pm 95% confidence interval. ANOVA followed by Tukey test was done, letters indicate significant differences among means (pvalue<0.001). Statistical analysis such as ANOVA followed by a Tukey's post hoc test and T-test were done in GraphPad Prism.

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4.2.3 Involvement of other CrRLK in osmotic signaling

In the previous section we have shown that the CrRLK FERONIA is acting on ROP6 signaling by modulation of PS localization at the PM. As explain in the introduction, the cell wall sensing pathway is mediated by different CrRLK1s. Among them, THESEUS appears central for regulation of rapid cell growth responses. Here, we would like to investigate is role during osmotic stimulation and determine if we can extend our results on FERONIA to other CrRLK1s.

THE1, a key signaling element in CWI is also required for the osmotically induced ROS accumulation

We used THE1 loss-of-function (*the1-1*) (Hematy et al., 2007) and a gain-of-function (*the1-4*) allele (Mertz et al., 2017) incubated in either resting or -0.75MPa solution. Compared to Col-0, loss of function THE1 has no increase in the osmotically induced ROS, whereas the gain of function the1-4 shows Col-0 like phenotype (Fig16-A). This indicates that proper functioning of the CWI via THE1 is needed for the osmotically induced ROS accumulation.

LPS complement THE1 loss of function phenotype but triggering THE1 pathway with RALF34 is dispensable for the PS localization

From our previous data we showed the relation between FER-PS-ROP6. We demonstrated that LPS add backs resulted in some *fer*-related phenotypes, such as the osmotically induced ROS, shape of the PC and flg22 response. Because of the reports showing that FER could regulate activity of other CrRLK1s, in this case, THE1, indirectly, by increasing the apoplastic pH, we have decided to check if LPS would have the same effect on THE1. Our data, shows that exogenous addition of LPS could partially rescue the loss of osmotically induced ROS accumulation in *the1.1* mutant (Fig17-A). Finally, because THE1 was described as a receptor for the RALF34 ligand, we used this peptide to stimulate THE1 pathway and check if this has an effect on the PS localization. It seems that RALF34 treatment doesn't have an effect on the PS localization since the PS bio sensor 2xPH^{EVECTIN} is unmodified by the treatment (Fig17-B). Taken together, our results show that LPS has a similar effect on THE1, as it has for FER but RALF34 peptide is dispensable for PS localization.

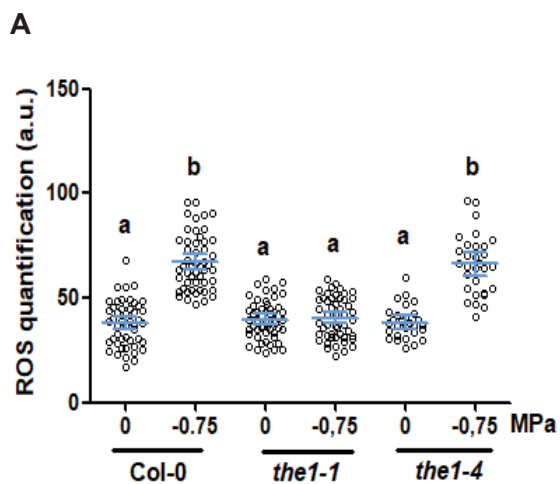


Figure 16: **THE1 is required in triggering the osmotic stress signaling.** (A) ROS quantification from DHE fluorescence signal of Col-0, *the1-1* and *the1-4* in control condition (0 MPa) or after 15 min of -0.75 MPa treatment.

Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p -value<0.001). n >18 from at least 2 biological replicates.

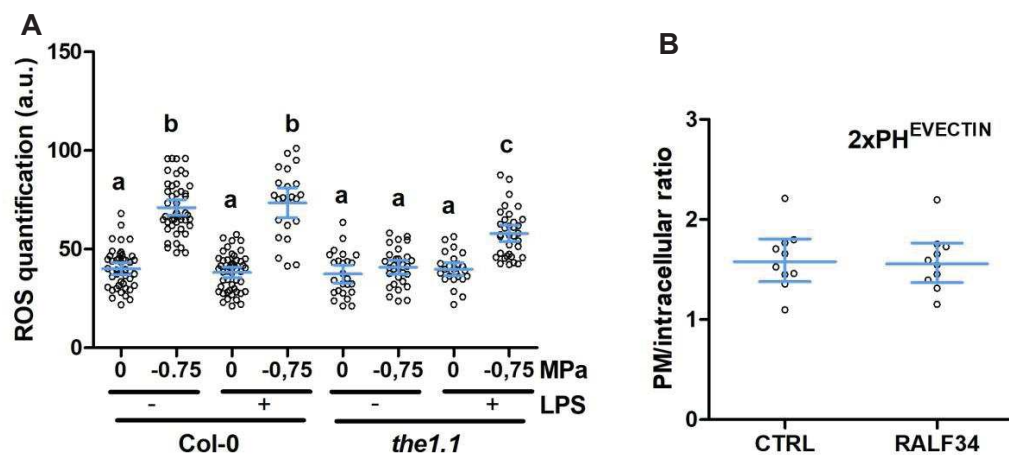


Figure 17: **Exogenous addition of PS can reverse *the1.1*-mutation osmotically induced ROS but THE1 stimulation by RALF34 has no effect on PS intracellular dynamics.** (A) ROS quantification from DHE fluorescence signal Col-0 and *the1.1* in control condition (0 MPa) or after 15 min of -0.75 MPa treatment with (+) or without (-) 1h LPS pretreatment. (B) Quantification of the PM/intracellular m-citrine signal ratio of 2xPH^EECTIN in control condition or after 1h pretreatment with RALF 34. Error bars correspond to a confidence interval at 95%. ANOVA followed by Tukey test, letters indicate significant differences among means (p-value<0.001). In (B) t-test indicate values differences are non significant.. n>20 from at least 2 biological replicates in (A) n=10 from one repeat in (B)

4.3 Discussion

In this chapter, we have demonstrated the link that exists between the CWI and the osmotic sensing. We found that cell wall perturbation interferes with osmotically dependent ROS accumulation. Both FER and THE seems to be needed for inducing osmotic signaling. But at least for FER, we found that do not act as sensor for the osmotic signal. Rather FER play on membrane composition and tune ROP signaling in plant. In the next section, I will discuss the interplay between cell wall perturbation, hypo/hyper osmotic stress and cell wall sensors interconnection.

Cell wall perturbation leads to abnormal osmotic signaling.

Our results showed that the cellulose assures normal osmotic stress signaling because mutants in the CESA complex (*kor*, *prc*, *rsw*) present modified ROS accumulation in response to the osmotic stress. Therefore, a normal functioning cell wall composition is required to insure proper CW mediated osmotic signaling. The perturbations in the cellulose composition by ISX seem to go via the ROP6 signaling pathway, more precisely the data showed that the ISX effect is upstream of ROP6, ROS produce by autoactive ROP6 is insensitive to ISX. Nevertheless, it is surprising that constitutively activated ROP6 doesn't respond to the ISX treatment in the same way as the co-treatment of ISX and -0.75MPa solution medium in Col-0. This could be due to the fact that genetically pre-activated ROP6 can't get more activated. Interesting result is the phenotype of *mur1.1* which show no differences for the osmotically induced ROS compared to the WT, meaning that the pectin organization in the cell wall is not important for this particular phenotype.

But, stresses reducing the strength of the cell wall should have effects similar to hypo osmotic stress since the high levels of turgor pressure prevalent in plant cells in combination with a weakened cell wall would also lead to plasma membrane stretch. This is what we can expect from plant with altered cell wall or treated with ISX. In this line it was found that the ISX effect on cell expansion were reversed by sorbitol in a THE dependent manner (Hamann et al., 2009; Engelsdorf et al., 2018). This insinuates that upon hypo osmotic stress triggered by ISX, THE1 sense the CW perturbation and trigger downstream responses such an increase in the ROS, which then by return initiate plethora of responses such as lignin deposition, gene expression, modulation of root growth. In response to CWD, as an early response ROS are produced in RbohF dependent manner, which in return leads to lignin deposition to reinforce the cell wall. This process is THE1 dependent (Denness et al., 2011). In alliance with this, our data also

indicates that THE1 modulate the osmotically induced ROS and even though the system used in Denness study differs from ours (they have used luminol for their ROS assays). Moreover, in our system we don't know by which mechanism THE1 act on the ROS accumulation upon hyperosmotic stress. Does THE1 act upstream of ROP6 or control ROP6 nanodomain formation is still unknown. Also, it is not impossible to imagine that THE1 act indirectly on ROP6 dependent osmotic pathway through another CrRLK1K.

Is THE1 regulated by FER in the osmotic cascade?

In our experiment *the1.1* show decrease in osmotically dependent ROS production and this defect is in many points similar to what was found with FER loss of function plant. This would suggest that a role for CrRLK1Ls in controlling membrane lipid composition. Nevertheless, those results are still preliminary and we have not rule out all the alternative explanation. Especially, it is not excluded that some inference between the CrRLK exist and therefore we are not fully sure that FER pathway is still functional in *the1.1*. THE1 and FER share great number of common processes but there is still molecular division and some specificity. It was shown that THE1 in comparison to FER is not involved in the PAMP-induced responses (Engelsdorf et al., 2018). FER inhibits the activity of the plasma membrane-localized H⁺-ATPase AHA2 in a RALF-dependent manner, thereby increasing the apoplastic pH and inhibiting growth (Haruta et al., 2014). This change in the apoplastic pH may also modulate the function of other CrRLK1Ls family members since THE1 appears to be incapable of binding to RALF34 at low pH but capable of binding at high pH (Gonneau et al., 2018). Thus, if the FER–RALF interaction indeed increases the apoplastic pH, the interaction of THE1 with RALF34 would be enabled, possibly modifying THE1 activity. This shows that at least FER could regulate THE1 and that availability of RALFs is an important determinant for activation of PTI or CWI signaling (Vaahtera et al., 2019). This might explain the partial recovery of the osmotically induced ROS of the *the1.1* mutant under LPS. If we imagine that FER and THE1 exist in a complex, disruption of one of the elements, in this case THE1 would result in a phenotype. Since our hypothesis is that PS act downstream of FER, the LPS add back assay can indeed complement the THE1 mutation by complementing the putative FER-THE1 complex. Also it is evident that stimulation of the THE1 pathway by its corresponding peptide RALF 34 has no effect on the PS localization. This could be explained with the notion that the PS localization is simply RALF34 independent. Possibly another unidentified RALF peptide that binds THE1 could be involved in the PS relocalization. Reports show some phenotype discrepancy between the receptor kinase mutant and its ligand mutant. This is particularly the case of the differences in lignin deposition between *the1.1* and *ralf34*. *the1.1* has decreased

levels of lignin deposition after ISX treatment where as *ralf34* lignification is unaltered compared to Col-0 (Gonneau et al., 2018).

Many aspects of the CW sensing and the osmotic signaling are common, but not the upstream signal. Moreover, with our experimental approach we can suggest that the CW sensing responds to both hypo and hyper osmotic stress but it is still unclear whether THE1 is directly involved in the osmotic signaling or indirectly by being regulated via FER.

5. General discussion and perspectives

As stated in this manuscript's objectives, the goal of my thesis was to explore and enlarge the molecular network of osmotic signaling. I aimed to uncover novel molecular actors involved in the perception and early signaling mechanisms of plant cells during osmotic stress. Furthermore, a signaling pathway based on functional or physical interactions of these actors and their dynamics within membrane nanodomains was described. And the role of these components in an integrated response to osmotic stress was evaluated.

In the course of my work, we identified new molecular actors in the osmotic signaling cascade. Starting from the accumulation of ROS upon hyperosmotic stimuli, we were able to describe that there are 2 major pathways for triggering the osmotically induced ROS. Upstream of them, a Rho GTP-ase, ROP6, has an essential regulatory role. We also discovered an activator of ROP6, GEF14 that specifically regulates ROP6 upon osmotic stimuli, but not other signals (such as ABA or flg22). Phosphatidylserine (PS) is needed to ensure normal osmotic signaling and it seems to act upstream of the activated ROP6. PS turnover is regulated by CrRLK, FER, which we believe coordinates the GTP-ase signaling in general. We were able to identify components of the cell wall and the cell wall integrity sensors such as FER and THE1 that are implicated in the osmotic signaling. It seems that the cell wall sensing is not a consequence of the osmotic signaling but more a joint interplay of the two. Our data showed that cellulose has a very important role in osmotic signaling since mutants in the cellulose synthase complex show important hyperosmotic phenotypes. Loss of a cell wall sensor THE1 is also impaired in the osmotically induced ROS but whether THE1 acts directly or indirectly through FER is still unclear.

How is Rboh regulated during osmotic signaling?

The effect of constant high ROS accumulation even in control conditions generated by ROP6-CA suggests that only activated ROP6 might be sufficient to activate Rboh. However, a point I would like to raise here is that there are many examples for different stimuli where Rboh can be activated without ROP6 (in case of PTI, CWD, ABA...)(Kwak et al., 2003; Jiao et al., 2013; Kadota et al., 2014; Drerup et al., 2013). That is why we investigated the involvement of known Rboh regulators such as BIK1, CPK5/6/11 and others (chapter1, 3.3.1). Mutants of *bik1*, *cpk5/6/11* have impaired osmotic induced ROS, which indicates that they are placed in the osmotic cascade. Kadota and colleagues described that BIK1 can phosphorylate, therefore activate RbohD upon flg22 perception and produce ROS. But, their immunoprecipitation assays also showed that BIK1 is able to constitutively but weakly associates with RbohD in

resting state as well. This indicates that RbohD might have steady-state phosphorylation that is needed for proper Rboh functioning and is regulated by BIK1. Even though their work was performed in a different system (shoot) and different stimuli (elicitors), this might explain the loss of ROS in the *bik1* mutants.

I have found that ROP6 is important for the “3D” organization of the Rboh on the PM in osmotic signaling. This is supported by the GTP-ROP6 data that has constitutive higher ROS accumulation and resides in PM domains even in resting conditions. Moreover, the ROP6 version that can't get targeted to domains doesn't produce ROS but if we claim that ROP6 recruits Rboh in nanodomains and render it active, the fact that Rboh still forms cluster in *rop6.2* upon osmotic stress come a bit paradoxally. This shows that making Rboh clusters per se in a membrane is not enough for inducing ROS. Probably other components, for example ROP6, are needed too.

During osmotic signaling that results in ROS accumulation RbohD is being “dragged” to domains by activated ROP6 together with RbohF which seems to presides in clusters (even though with our set up, we can't say for sure that they are the same clusters). This goes in favor of the hypothesis that Rboh forms heteromers. Results from osmotically induced ROS in the *rbohD/rbohF* double mutants show genetic interaction (Martiniere et al., 2019). Changing the localization of Rboh within the PM might be a trigger for its “other” regulators such as BIK1 and CPKs and they might also change localization “chasing” Rboh. It is not excluded that some of them might reside in the same cluster as ROP6/Rboh as a tool to modulate the phosphorylation status of Rboh. Further studies exploring the protein dynamic of Rboh in *bik1* or *cpk* mutant background would be helpful. In conclusion, we could imagine that all Rboh regulating proteins are contribute to a functional signaling platform and could be in the same place in space and time upon activation. This is somehow an exciting opportunity to explore Rboh regulation in terms of PAMP signaling and osmotic signaling. Preliminary data show that ROP6 is also crucial for flg22 and ABA-induced ROS accumulation. We could imagine that disruption in ROP6 has an impact on Rboh therefore loss of ROS response upon flg22 and ABA. Indeed, Zhai et al., 2018 noticed that transcripts levels of RbohD and RbohF in *rop6.2* mutant is decreased. This might be a trivial explanation of the loss of phenotype we see in *rop6.2* with different stimuli.

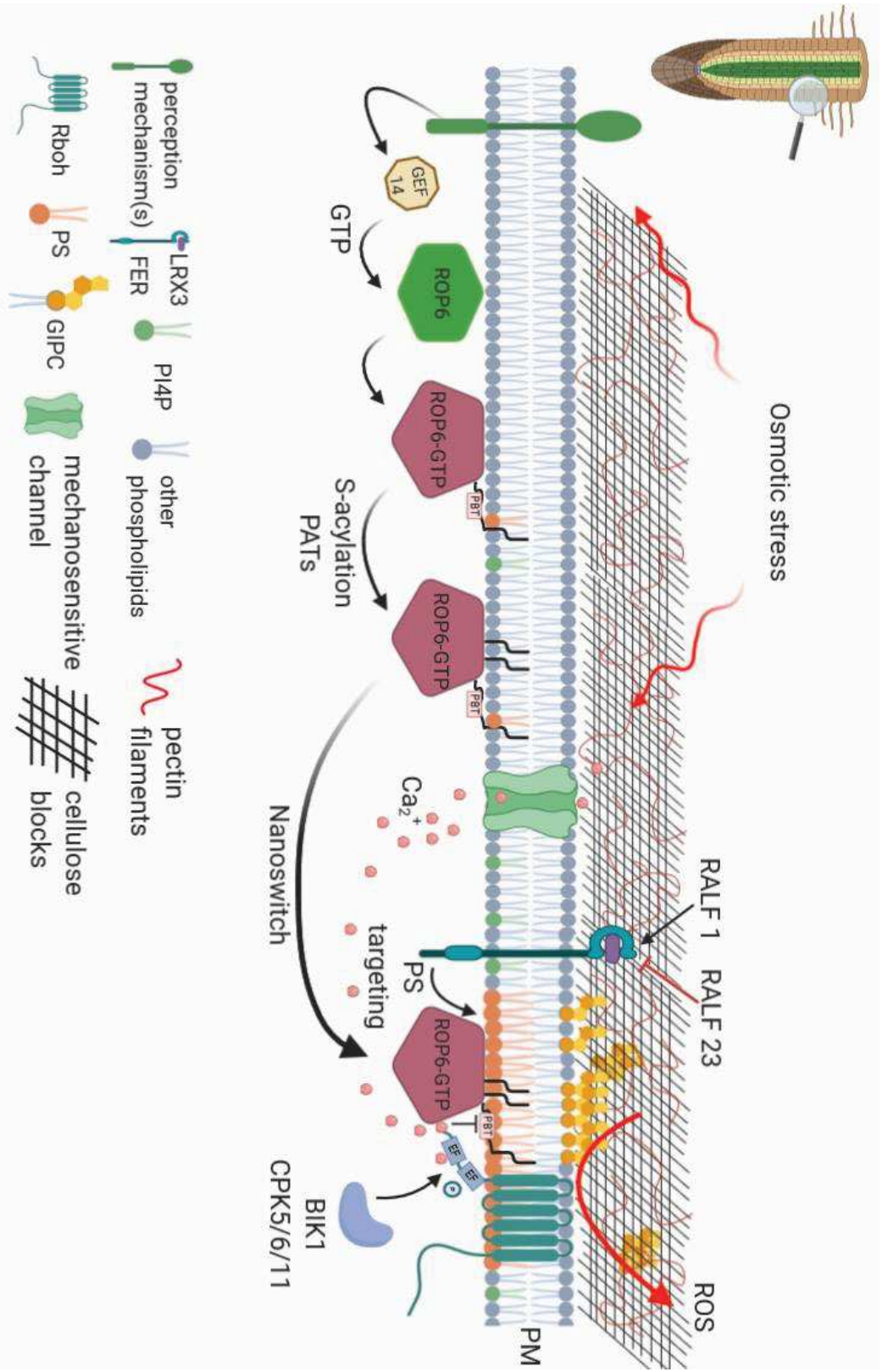


Figure 18: Hypothetical model for ROP6/Rboh activation in nanodomains

Upon the putative osmotic stress perception mechanism(s), GEF14 could switch ROP6-GDP into ROP6-GTP. Posttranslational S-Acylation targets ROP6-GTP into PS nanodomains together with Rboh. In the nanodomains PS is represented at the inner PM leaflet and GIPC on the outer leaflet. This domain could be mirrored by specific domain in the CW. PS localization and domain targeting is dependent on FER. Ca²⁺ generated from mechanosensitive channels might act as a direct competition between the PS-ROP6 binding, this having a possible negative feedback. Nevertheless, Ca²⁺ could positively regulate Rboh together with BIK1 and CPKs that could phosphorylate Rboh.

ROP6 regulate osmotic signaling via PS nanodomains

In the first chapter of this manuscript, I demonstrated the ROP6 has a crucial role in the osmotic signaling, harbored in specialized osmospecific nanodomains. The unique properties of protein segregation in nanodomains within the membrane emerges as an essential feature in several signaling pathways. I described that upon hyperosmotic stress ROP6 co-segregates, together with its effector Rboh, in PM domains. Because ROP6 is also targeted in nanodomains in a PS-dependent manner in auxin signaling context (Platre et al., 2019), our hypothesis is that several domains exist depending on the upstream signal and the constituents in the domain contribute to the specificity. This aspect is discussed in 3.2.2. But what is the function of GTP-ase nanoclustering? In general terms, the partitioning of proteins in PM domains is viewed as their localization into “signaling platforms”. What is behind the concept of “signaling platform” is often ambiguous, but the main idea is that these domains will locally concentrate protein partners or complexes, thereby contributing to signaling. In cases of ROP6 partitioning, it is relatively easy to understand how such a concept could be involved in osmotic signaling. Indeed, ROP6 nanopatterning follows a switch-like behavior (out of the cluster in resting condition, inside the cluster in activated condition). Therefore, ROP6 could meet its effectors only when present in nanodomains. Indeed, after osmotic shock but not auxin, Rboh is also recruited in the same nanocluster as ROP6 (Fig18). On the plasma membrane outer leaflet of plant cells, GIPC (glycosylinositolphosphoryl-ceramides) are the most abundant lipids (Gronnier et al., 2016). They have large head groups that are predicted to extend into the cell wall and may therefore have a limited diffusion. In addition, they are sphingolipids with very long chains, which could be involved in transbilayer coupling with inner leaflet phospholipids. One could speculate that this system may allow the formation of stable PS nanoclusters in the cytosolic plasma membrane leaflet (Fig18).

In addition, ROP6 accumulation into PM nanodomains also includes the notion of “clustering”. In the case of K-Ras, nanoclusters contain ~6-7 Ras proteins per nanocluster (Janosi et al., 2012). This is important because Ras dimerization is a prerequisite for signaling. Similarly, it could be possible that ROP6 dimerization or higher-order complex could be important for signaling. However, as it was discussed in Chapter 1 (3.3.1) the mechanism by which the osmospecific Rboh/ROP6 complex gets into the nanodomain needs to be better understood.

How is ROP6 regulated?

ROPs possess a polybasic region (PBR) which interacts through electrostatic interactions with anionic phospholipids. However, ROP6 nanoclustering is a fast response (within 5 minutes of hyperosmotic treatment). It is therefore unlikely that an increase of PS into nanoclusters would be the trigger for ROP6 clustering as this would imply that proteins involved in PS clustering are very early targets of the osmotic stimuli (earlier than ROP6). In addition, there is no PS relocalization upon osmotic nor auxin stimulation. Thus, PS domains appear to be stimulus independent.

Calcium signaling is interconnected with ROP polarization since calcium concentration gradient correlates with ROP activity in polar tip growth (Himschoot et al., 2015). Moreover, PS has a particular affinity with calcium (Martin-Molina et al., 2012) and in turn, might act as a competitor for ROP6 retention time in the clusters. This is supported by the fact that there is a transient calcium increase upon osmotic stimuli and indeed the lipid binding could be disrupted. On another side, Ca^{2+} is known to be a positive regulator of Rboh so based on this it might have different regulatory roles for different components from the same domain.

As for a potential activator of ROP6 during osmotic signaling, GEF14 constitutes a very promising candidate since is necessary for osmotically induced ROS. In addition, seems that GEF14 holds the specificity for the osmotic signal. In other words, GEF14 might be the factor responsible to activate ROP6 specifically upon osmotic signaling. Proper interaction study is needed to confirm the actual interaction between ROP6 and GEF14 and most importantly we need to know if GEF14 acts upstream of ROP6. For this the ROP6 auto active line crossed in *gef14* mutant will be helpful. Next step would be to investigate if GEF14 modulates ROP6 clustering in osmotic stress. For this, a TIRF imaging of *gef14xGFP-ROP* should be done.

The discovery that GEF14 might be the upstream activator of ROP6 in osmotic signaling could be exciting feature to try to understand better specific nanodomains in plants. Depending of the environmental stimuli, ABA, osmotic, auxin, flg22, different GEFs would activate ROP6 and this would target it to its proper stimuli dependent nanodomain which most probably different lipid composition. This would explain the role of a single ROP isoform being involved in different signal pathways (Fig 19). Upon activation (by GEF14), ROP6 is transiently S-acylated on two cysteine residues, which are required for its function and for its targeting to DRM (Sorek et al., 2010). This is in accordance with our data showing that ROP6 is getting immobilized when activated from osmotic stress. Modeling suggests that both acylated cysteines (C21 and C156) are located in the GTPase domain and are expected to be buried when ROP6 is not activated (Sorek et al., 2010).

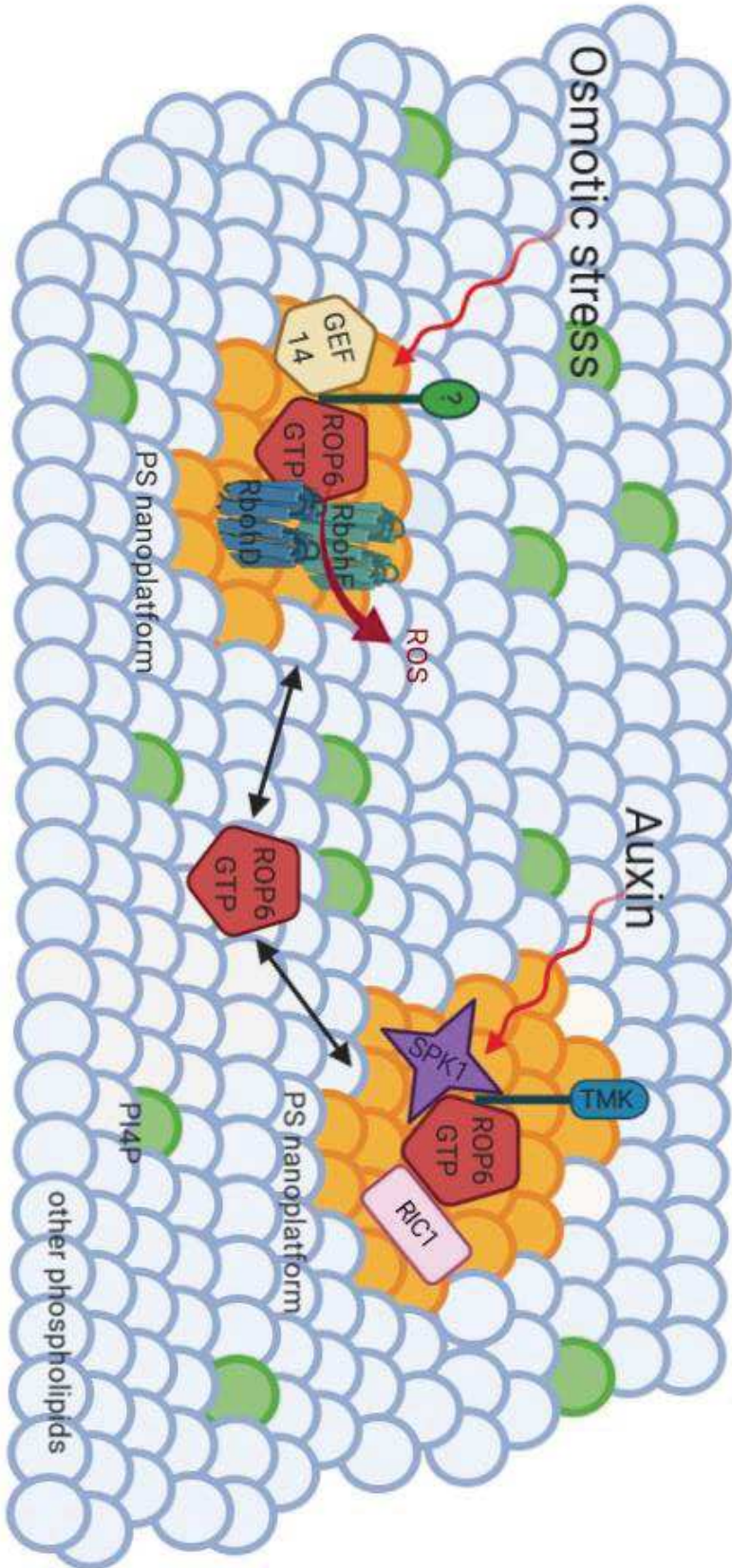


Figure 19: Top view over the PM and osmo-specific and auxin-specific nanodomains

Schematic representation of an osmospecific cluster where there is GEF14 as specific activator of ROP6 upon osmotic stress, unknown perception components (?) and the two Rboh isoforms, which are ROP6 effectors. On the side is presented another cluster, this time specific for auxin signaling with the auxin receptor TMK, specific GEF (SPK1), activated ROP6 and its effector RIC1. This model explained how ROP6 can be present in two different PS domains and how these domains can produce stimuli specific responses.

This goes in accordance with our data when ROP6 C21 and C156 mutated into alanine can't target ROP6 to nanodomains and can't produce ROS. However, Sorek et al., proposed that upon activation, the large conformational changes induced by GTP binding may expose the two cysteines to protein S-acyl transferases (PATs), which in turn would modify ROP6 acylation status and likely its localization into nanoclusters. Therefore, it would be interesting to screen for the possible PAT that might be involved in ROP6 clustering upon osmotic signaling. Then, we could think if there is one PAT specific for one signal? What is their localization in terms of membrane spatiality, are they nanodomain recruited? These aspects need further exploration.

How FER can regulate PS nanodomains?

Our data presented in chapter 2 clearly indicate that a receptor kinase FERONIA could regulate the localization of PS and by this probably regulates the ROP signaling. But the mechanism by which this is happening is still unclear. One way to explain this is to connect the FER regulation and the PS synthesis and targeting to the PM. In yeast and mammalian cells, PS bypasses classical vesicular trafficking and rather directly translocates from the ER to the PM, at membrane contact sites (MCSs). MCSs are static microdomains where membranes from two different organelles are brought together and stabilize by tethering proteins (Scorrano et al., 2019). MCSs are major sites of lipid flow and are particularly important to control the localization of nearly all anionic lipids (Wu et al., 2018). Lipid transfer proteins present at MCS are able to transport lipids between two membranes extracting the lipid from the donor membrane, transferring it through the cytosol and inserting it in the acceptor membrane. Notably, proteins from the oxysterol-binding related protein (ORP) family localize at MCS via interactions with suppressor of choline sensitivity Scs2p (VAPS in animal cells) in the ER and PIPs at the PM (Antonny et al., 2018, Wu et al., 2018). Several ORPs transfer PS (among other lipids) from the ER to the PM and then counter transport PI4P from the PM back to the ER (Fig 20). The PI4P phosphatase SAC1, an ER-resident protein, hydrolyses the incoming PI4P, thereby maintaining the PI4P gradient between the PM and ER membrane and fueling PS export from the ER (Antonny et al., 2018). PS transfer at MCS has not been described so far in plants but ORP proteins are conserved in plant genomes.

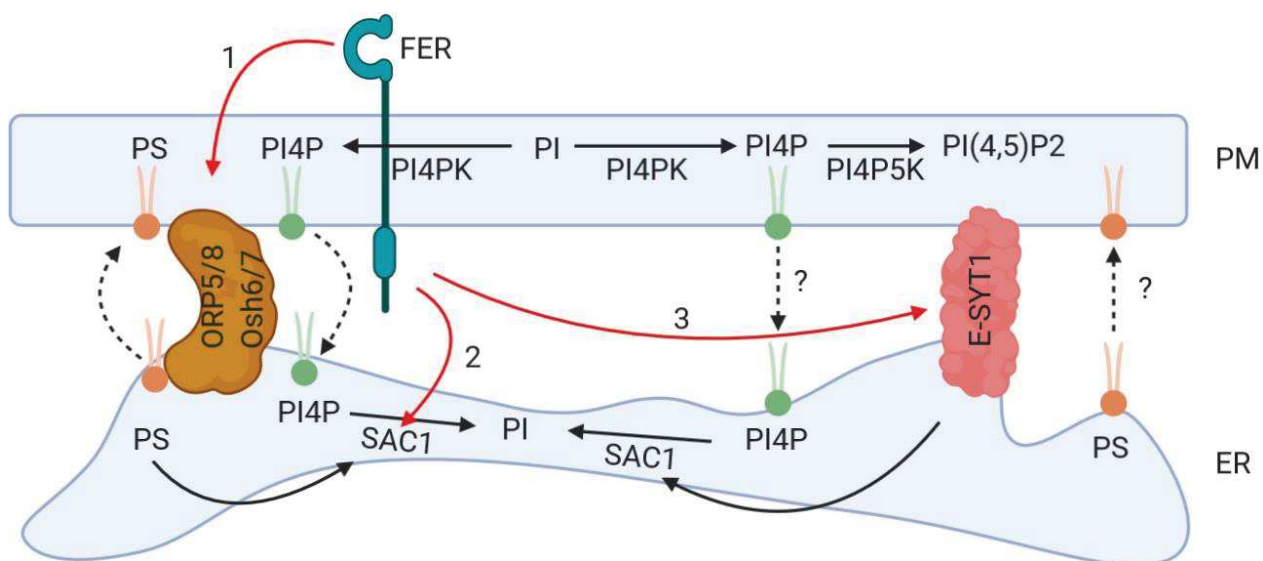


Figure 20: Lipid transfer at ER-PM contact site and possible FER regulation of the process

Mammalian ORP5/8 and yeast OSH6/7 are ER contact site localized proteins that exchange PS and PI4P between ER and PM. At the ER, PS activates SAC1 to dephosphorylate and replenish PI. This constitutes a feedback mechanism, which PS regulates PS production and exchange (dashed lines). In mammalian cells, ER-localizes E-SYT1 binds PI(4,5)P2 and the PM, thereby tethering the ER to the Pm and allowing SAC1 to decrease PM PI4P and PI(4,5)P2. One of the possible actions of FER in this process is regulating the ORP homolog proteins in plants (1), acting directly on SAC1 resulting in misslocalization of PI4P and or PS (2) or regulating SYT1 plants homologues.

Having all this in mind, we can hypothesize that FER could regulate the activity of some lipid transfer protein and thus can regulate the PS-PI4P counter transport in plants (even though counter transport has not been yet described). This can be done with its kinase activity by possibly phosphorylating proteins involved in PS PM targeting or by its scaffolding activity, as it is the case for the PTI (Stegmann et al., 2019). As there is a tight correlation between PI4P and PS at ER contact sites in yeast, FER might modulate this homeostasis in plants. To verify this hypothesis in plants first we should look how is the behavior of a Pi4P biosensor in *fer* loss of function roots or by using RALF1 or RALF 23 to stimulate or inhibit FER pathway. If indeed there are opposing differences in terms of cellular localization between PS and PI4P then we could think, in terms of kinetics, which lipid is the cause and which is the consequence of that putative counter-transport. Does PS internalization is an early event which leads to Pi4P overaccumulation on the membrane, or it is because of the PI4P targeting to the PM, PS gets internalized? We could also imagine a more indirect role of FER and that would be via SAC1, which would mean that FER as a receptor kinase could regulate the activity of a phosphatase. As I mentioned, the lipid exchange in plants is still unclear but the PM-ER-MCS are central for many cellular processes, such as cellular immune responses, viral movement at plasmodesmata, stabilization of the cortical ER network, endocytosis, membrane integrity, pollen, seed and root hair development (Kim et al., 2016, Levy et al., 2015, Lewis et al., 2010, Schapire et al., 2008). Synaptotagmin 1 (SYT1) is a tethering protein in the ER-PM-MCS which can directly interact with anionic lipids. The mammalian E-SYT1 is able in vitro to trigger glycerolipids exchanges between liposomes (Schauder et al., 2014). E-SYTs contain C2 domains that bind anionic phospholipids from the PM in a Ca^{2+} dependent manner (Yu et al., 2016). With the same rationale as for the ORPs, FER might regulate SYT1 activity. I have performed some preliminary experiments with the *sy1-1* mutant which showed no osmotically induced ROS. However, these data need confirming and further characterization.

FER at the interface between osmotic signaling and cell wall sensing

During the process of cell elongation, we could postulate that the changes in cell volume, initially perceived, by the cell wall are translated at the membrane by the PS domains. Modifications in the cell wall integrity have an impact on the microenvironment on the PM in terms of protein and lipid composition. The cell wall integrity can modify domains formation of PM proteins (Danek et al., 2020, McKenna et al., 2019). One could imagine that PS nanoclusters mirror nano environments from the cell wall. In other words, due to the stimulus, the cell wall would, most probably, as the PM, change its composition which would correspond

to the clusters on the PM. In between these putative cell wall nanodomains and PM nanodomains, FER through LRX might be the linking bond. One example further supporting this idea is that since FER was shown to bind to pectin *in vitro*, at least the pectin composition around FER would differ from the rest of the cell wall. In summary, FER could be a rheostat for PS domains that themselves in return can be modulators of the downstream signaling.

Furthermore, a recent study by Haas et al.2020 showed that pectin homogalacturonan forms nanofilaments within the cell wall which supports the possibility of interconnection of nano environments between CW and PM. Therefore it would be interesting to determine if indeed FER, PS and ROP6 are belonging to the same nanostructures and if there is specific CW nanoorganization in the vicinity. These domains could be the very early signaling compounds for many pathways like auxin, ABA, flg22, or osmotic.

When discussing the aspect of regular cellular processes such as cell elongation or environmental stress, we have to keep in mind that modifications in the cell wall might have many other consequences on the cell besides initiating cell wall integrity maintenance. Loosened cell wall might cause bigger cell deformation and this in return can act on the ROS production. In this line, our results show that the cellulose composition of the cell wall is crucial for osmotic signaling. The cellulose mutation in plants has pleiotropic effects but it is well described that THE1 is activated in these particular conditions. THE1 is the cell wall sensor upon cell elongation but in osmotic signaling, it appears it has a more indirect role. More precise characterization of THE1 is needed to determine if THE1 can act directly on the PS localization, either THE1 is controlled by FER by being in complex or maybe both? We still don't know if THE1 can be recruited in nanodomains upon osmotic signaling and if the THE1 pathway is ROP6 dependent and this is one aspect that needs investigating. Cross between the1.1 and ROP6-CA would be a good starting tool to see if THE1 is directly involved in the osmotic signaling cascade.

Finally, these two major cellular processes such as CW sensing and osmotic signaling share common molecular actors but lead to coordinated different responses. What does that mean for the cell? In the course of growth and development, the cell has to elongate in order to grow. The cell elongation is being carefully monitored by the cell wall integrity maintenance machinery. Extracellular change in the osmolarity is a closely related phenomenon to the cell wall disruption therefore an elongating cell needs to be more sensitive to the outside change in osmolarity, ion concentration, or even on a larger scale, water availability. This might be the reason why these two mechanistic processes need very fine coordination.

To conclude, ROP nanoclustering may have several critical functions for signaling, including signal transmission via an analog-digital-analog conversion relay but also signal compartmentalization between different ROPs (which have otherwise very similar sequences and bind similar effectors *in vitro*, but not *in vivo*). ROP nanocluster formation could therefore be a tool to generate both high fidelity and exquisite specificity during signal transduction. Using a combination of high resolution in planta imaging developed during my PhD, it will be possible in the future to test these different concepts and interrogate their importance in plant development and physiology.

6. Material and Methods

Because most of the results from my work are expressed in a form of research article the experimental part has already been described in the corresponding articles (pages: 58-59-60-61-151-152-153)

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8. Résumé en français

Introduction:

L'eau est l'un des facteurs abiotiques les plus limitants pour la croissance et le développement des plantes. Au niveau de la plante entière, en réponse au stress hydrique, les plantes réduisent leur cycle de vie (Sakamoto et al., 2008), limitent leur évapotranspiration par abscission foliaire (Sakamoto et al., 2008), et peuvent même modifier leur architecture racinaire afin d'optimiser l'alimentation en eau (Deak et Malamy, 2005). Ces réponses sont considérées comme des réponses à long terme produites après plusieurs jours. D'autres types de réponses se produisent plus rapidement, comme l'ajustement de la forme et de la croissance des cellules (Craddock et al., 2012) ou les modifications des propriétés de la paroi cellulaire qui conduisent à un ajustement osmotique, permettant de faciliter l'absorption d'eau dans les cellules (Dinneny et al., 2015).

D'autres types de réponses rapides surviennent quelques minutes après la perception du stress osmotique. Il s'agit par exemple de la fermeture des stomates (Islam et al., 2016) ou la modification de l'hydraulique racinaire (Boursiac et al., 2005). Enfin, des phénomènes cellulaires concernant la dynamique des membranes sont également induits. La modification du mécanisme d'endocytose est considérée comme l'une des réponses les plus rapides en réponse à un signal osmotique. Seulement quelques dizaines de minutes après l'application d'un traitement osmotique (chlorure de sodium ou mannitol), l'équilibre entre endocytose et exocytose est ajusté, probablement en vue de maintenir l'intégrité de la membrane végétale pendant l'ajustement du volume cellulaire (Luu et al., 2012; Zwiewka et al., 2015). Toutefois, en amont de toutes ces réponses, les plantes ont besoin de percevoir et de transduire le signal hydrique. Les mécanismes moléculaires sous-jacents sont à l'heure actuelle mal connus et ce principalement car le signal hydrique est multifactoriel. Au niveau d'un sol, la diminution du potentiel hydrique réduit le gradient osmotique entre l'extérieur et l'intérieur de la cellule, induisant d'abord une diminution de la turgescence cellulaire. Dès lors que la turgescence est nulle, un efflux d'eau va modifier drastiquement le volume de la cellule, il s'agit du phénomène de plasmolyse. Toutefois, le stimulus osmotique n'est pas le seul composant du signal hydrique. En effet, les propriétés mécaniques du sol changent en fonction de son humidité. Ce changement mécanique au voisinage de la racine pourrait également être perçu par les cellules végétales. De plus, en période de sécheresse, la plante perçoit une pénurie d'eau mais pas seulement. La rareté des nutriments est un effet secondaire du déficit hydrique, car l'eau véhicule les ions nécessaires à la croissance des végétaux. Par conséquent, il est évident que différents mécanismes de détection sont activés dans les plantes soumises à un stress hydrique. Pour simplifier l'aspect multifactoriel du signal hydrique, nous avons décidé de nous concentrer, pour ma thèse, sur le signal osmotique.

Perception osmotique

Les modifications de l'équilibre osmotique ont un effet important sur la physiologie végétale. Ces changements surviennent lors de nombreux stress abiotiques tels que le froid ou le stress hydrique mais aussi lors de la croissance et du développement des plantes (par exemple l'expansion cellulaire ou la germination des graines). Toutefois, malgré les nombreux exemples lors desquels le déséquilibre osmotique déclenche de vastes réponses, les mécanismes de détection des stimuli osmotiques sont encore largement inconnus. Néanmoins un certain nombre de systèmes de signalisation ont été décrits.

Si nous considérons les propriétés structurales des protéines transmembranaires, il apparaît qu'elles peuvent une fois incorporées dans la MP directement sentir les changements d'osmolarité à l'intérieur et à l'extérieur de la cellule. A partir des connaissances initiales issues de la levure, il a été identifié une histidine kinase, ATHK1, agissant comme un osmosenseur végétal putatif. ATHK1 présente une similitude structurelle avec l'osmosenseur de levure SLN1. Par ailleurs, lors d'un choc osmotique (hypo ou hyper), la tension des membranes varie, avant toute déformation du protoplaste cellulaire. Les canaux mécanosensibles (MS) modifient leur conductivité aux ions en réponse aux changements de tension de la membrane et peuvent donc convertir les signaux mécaniques en flux ioniques. L'un des canaux mécanosensibles perméables au calcium les mieux caractérisés qui joue un rôle dans la signalisation osmotique est OSCA1. Le mutant *osca1* montre une altération de la signalisation calcium dans les cellules de garde et des racines en réponse au signal osmotique.

De plus, ce même mutant montre une réponse atténuée de la régulation de la transpiration ainsi que de la croissance des racines en réponse au stress osmotique. Ces résultats montrent le rôle central d'OSCA1 dans la signalisation osmotique. En plus de son effet sur la tension des membranes, le traitement osmotique induit une déformation cellulaire. Cela a un effet direct sur le continuum qui existe entre la paroi cellulaire et la membrane plasmique. Par conséquent, certaines protéines ancrées dans la membrane plasmique sont soupçonnées de percevoir ces changements. Il s'agit en particulier des kinases de type RLK1 de *Catharanthus roseus* (CrRLK1L) qui ont été largement étudiées pour leur rôle dans la détection du statut de la paroi. THESEUS (THE) et FERONIA (FER) sont parmi les CrRLK1L les plus étudiés. Historiquement, l'étude d'une population EMS portant la mutation PROCUST / Cellulose synthase 6 (*prc/cesa6*) a été criblée pour la perte de phénotype « petit hypocotyle ». Il a été constaté qu'une mutation dans THE1 atténue considérablement le défaut d'élongation de l'hypocotyle et la production de lignine ectopique de *prc1*, suggérant un rôle dans l'inhibition de l'expansion cellulaire lorsque les défauts de la paroi sont détectés (Hématy et al., 2007). FER, d'autre part, a un profil fonctionnel beaucoup plus large et semble servir de régulateur maître pour de nombreux processus, dont la fécondation, la croissance des plantes, la signalisation hormonale, la mécano-détection et la défense des plantes (Liet al., 2016; Liao et al., 2017; Dunser et al., 2019). FER est nécessaire au maintien d'une distribution spatiale stable de l'expansion cellulaire dans la zone d'élongation des racines et joue un rôle clé dans la médiation de la signalisation mécanique en amont des changements de Ca_2^+ cytosolique (Shih et al., 2014). De plus, Feng et al 2018 ont montré que la salinité

provoque un ramollissement de la paroi et que FER est nécessaire pour détecter ces défauts. Comme FER et THE ont tous deux des domaines extracellulaires de type malectine, ils pourraient détecter l'état de la paroi en se liant directement aux glucides ou glycoprotéines dérivés de la paroi (Cheung et Wu, 2011).

Mais récemment, un nouveau mécanisme de détection de la paroi cellulaire est apparu, mettant en jeu d'une part des extensines de la paroi cellulaire à domaine LRR (leucine riche region) (LRX) ainsi que des peptides apoplastiques de la famille des Rapid Alcalinisation Factor (RALF) mais aussi le co-récepteur LGG1. En résumé, plusieurs hypothèses peuvent coexister lorsque l'on parle d'osmoperception végétale. Un signal mécanique généré à partir d'événements membranaires ou de perturbations du continuum PM-CW pourrait contribuer à la perception du signal osmotique. Ce qui ressort également de ces études, c'est qu'une multitude de mécanismes de détection osmotique coexistent dans les cellules. Cela n'est probablement pas surprenant au vu du rôle primordial que la pression hydrostatique exerce sur la physiologie et le développement des plantes. Une façon de décrire de nouveaux mécanismes moléculaires dans la voie de signalisation osmotique chez les plantes consiste à utiliser une approche ascendante à partir de l'activation de la transduction du signal. C'est la stratégie suivie par l'équipe et je détaillerai dans la section suivante ce que l'on sait du signal osmotique et des messagers secondaires cellulaires. Transduction du signal de contrainte osmotique

La base de la transduction du signal est le transfert et l'intégration de ce signal à travers une cellule. Nous décrivons ici quelques acteurs cruciaux dans la série d'événements moléculaires qui se produisent entre la perception du signal osmotique et les réponses cellulaires en aval. L'activité protonique (H^+), l'ion calcium, les lipides de signalisation tels que l'acide phosphatidique et les protéines kinases de stress osmotique sont des messagers secondaires importants. Parce que je suis limitée en termes de pages, je me concentrerai sur les espèces réactives de l'oxygène (ROS) qui s'accumulent en quelques dizaines de minutes après un stress osmotique et qui sont très étroitement associés à la signalisation osmotique dans les racines d'*Arabidopsis*. Par exemple, Rejeb et al., 2015 ont rapporté que le peroxyde d'hydrogène (H_2O_2), une des molécules clés de la signalisation ROS produite lors du signal osmotique, induisait une accumulation de proline. La proline est le premier acide aminé à s'accumuler dans les plantes soumises à un stress hydrique. Elle permet aux cellules de rétablir une pression osmotique interne suffisante. Toutefois, les ROS sont également associés à des réponses rapides des organes. La régulation de la conductivité hydrique des racines permet de limiter la perte en eau lors d'un stress salin ou osmotique. L' H_2O_2 peut inhiber la perméabilité à l'eau des racines lorsqu'il est ajouté de manière exogène dans le système, tout comme le fait le signal salin ou osmotique. Boursiac et al., 2008 ont étudié le rôle potentiel de H_2O_2 sur les canaux à eau des racines, les aquaporines, et ont découvert que H_2O_2 n'agit pas sur les aquaporines par un effet oxydant direct, ni même sur la régulation transcriptionnelle ou traductionnelle des aquaporines. En réalité l' H_2O_2 agit sur l'internalisation des aquaporines depuis la membrane plasmique (Wudick et al., 2015 mol plant). Martinière et al., 2019 ont montré que les ROS qui sont produits dans des conditions hyperosmotiques régulent l'internalisation des membranes et agissent en particulier sur l'endocytose de PIP2;1, une aquaporine particulièrement abondante dans les racines. Ces résultats

démontrent le rôle prépondérant des ROS dans la réponse des cellules exposées à un signal osmotique. Mais comme les ROS sont-ils produits en réponse à ce signal? Les enzymes homologues des NOX chez les plantes (Rboh) sont des acteurs majeurs de la signalisation ROS. Elles sont bien connues pour leurs rôles dans la signalisation pathogène et en réponse à la phytohormone ABA. De manière attendue, les Rbohs sont aussi impliquées dans la signalisation osmotique, en particulier les isoformes D et F. Toutefois, Martiniere et al 2019 ont identifié un phénomène supplémentaire dans la signalisation ROS. En effet, le fer réduit (Fe_2^+) est également impliqué dans l'accumulation des ROS. Ce système original de signalisation n'est pas caractérisé de manière moléculaire mais semble nécessiter une réduction transitoire de l'apoplaste des cellules, probablement grâce à de l'ascorbate. Les mécanismes de production de ROS étant caractérisés, il s'agit désormais de comprendre comment le signal osmotique les active. La régulation de l'activité des Rboh est assez bien connue. Il apparaît que de multiples voies indépendantes sont en jeu. Par exemple, les Rboh ont des motifs de type EF hand dans leurs régions N-terminales et, de fait, elles peuvent être directement régulées par le Ca_2^+ . La mutation des motifs EF, décrite par Ogasawara et al., 2008, entraîne une diminution de la production de ROS. Une analyse structurale et biochimique a montré qu'OsRbohB a deux motifs de type EF, mais que le Ca_2^+ se lie uniquement au premier (Oda et al., 2010). De plus, deux types de kinases activées par le calcium sont connues pour phosphoryler les Rbohs. D'une part, les CPK sont des régulateurs importants de Rbohs en particulier les isoformes 5, 6 et 11. D'autre part, certaines CIPK sont également connues pour cibler le domaine N-terminal de Rboh. Dans la réponse immunitaire innée (Pathogen trigger immunity, PTI), BAK1 s'associe et active BIK1. Li et al., 2014 ont décrit que BIK1 peut directement phosphoryler RbohD lors de la perception du peptide bactérien flg22 (agissant comme un éliciteur de défense). Chez les mammifères, l'un des principaux régulateurs des NOX est une petite GTP-ase, Rac2. Des homologues de RAC existent chez les plantes et ces homologues ont été largement caractérisés comme des régulateurs potentiels de l'activité des Rboh. Par exemple, la petite GTPase de riz, RAC1, a été décrite comme un régulateur positif d'OsRbohB (Oda et al., 2010). De plus, des mutations dominantes positives de l'isoforme ROP6 (ROP6-CA) d'Arabidopsis induisent de manière constitutive des valeurs élevées de ROS dans le poil racinaire (Sorek et al., 2010). L'accumulation de ROS exerce en retour un effet sur la morphologie des poils racinaires. Par ailleurs, la sur-expression de ROP6-CA bloque l'endocytose. Cet effet est conforme aux résultats montrant que l'expression ectopique de RAC10 peut modifier le cyclage membranaire (Bloch et al., 2005). En conclusion, tous les exemples ci-dessus montrent que Rboh est régulée par différents facteurs. Parmi eux, les petites GTPase sont particulièrement intéressantes. Effectivement, en plus de leur effet sur la régulation du Rboh, elles ont été décrites comme des régulateurs négatifs de l'endocytose (Chen et al 2012, Sorek 2010). De fait, nous savons que l'une des réponses cellulaires au signal osmotique la plus rapide est la modification de l'endocytose. Par conséquent, la famille des protéines ROP constitue un candidat très prometteur de la régulation des Rboh dans le cadre du signal osmotique.

Objectifs:

Depuis ces dernières années, les acteurs moléculaires qui interviennent dans la cascade de signalisation osmotique commencent à être élucidés. Néanmoins, de multiples mécanismes de perception semblent coexister chez les plantes. Les ROS, agissant comme messagers secondaires semblent jouer un rôle crucial dans la transduction du signal osmotique, ce qui en fait d'excellent marqueurs pour étudier les mécanismes en amont. La famille des gènes Rboh est essentielle pour de nombreuses voies de signalisation cellulaire depuis la croissance et le développement jusqu'à la signalisation en réponse aux pathogènes. La manière dont les Rboh sont activées par le signal osmotique reste totalement inexplorée. Par conséquent, l'objectif principal de ma thèse est de déchiffrer les mécanismes de signalisation précoce déclenchés après un signal hyperosmotique. Cela comprend en particulier la régulation en amont des Rbohs. Par une stratégie ascendante («bottom up»), nous avons d'abord utilisé les ROS induits osmotiquement pour trouver d'éventuels candidats régulateurs de Rboh. Nous avons tout d'abord examiné les petites GTPases de type Rho des plantes (ROP). L'isoforme ROP6 régule à la fois les mécanismes de génération des ROS induits par le signal osmotique mais également une partie de la réponse de la plante à la stimulation osmotique. Nous avons également examiné l'organisation spatiale de ROP6 au sein de la membrane et démontré que la signalisation osmotique est étroitement régulée par la répartition de ROP6 dans les nanodomains membranaires. Nous nous sommes ensuite demandé comment la ROP6 pourrait elle-même être activée. ROP6 peut exister entre une forme active et inactive, une caractéristique commune pour les petites GTPases. Dans ce schéma, les facteurs d'échange de nucléotides guanine (GEF) permettent de favoriser le passage des ROPs de leurs formes inactive à actives. Nous avons trouvé un activateur, GEF14, qui pourrait être un activateur direct de ROP6. Plus important encore, GEF14 semble être spécifiquement nécessaire pour la signalisation osmotique. Classiquement, les GEF et les ROP agissent en aval de récepteur membranaires. Nous avons donc utilisé une approche « gène candidat » pour étudier les implications possibles de certains membres de la famille CrRLK1Ls. Ces récepteurs kinases membranaires sont connus pour participer à la perception du statut de la paroi cellulaire, ce dernier étant fortement impacté pendant le stress hyperosmotique.

Résumé des résultats**Les nanodomains ROP6 assurent la spécificité du signal lors du stress osmotique**

Les petites GTPases sont des acteurs importants de la signalisation membranaire, agissant directement en aval de récepteur kinase. Il a été montré que les ROP ne sont pas réparties de manière homogène au sein de la membrane plasmique. En fait, une forme constitutivement active de ROP6, générée en mutant sa Gly15 en Val s'accumule dans la fraction de membrane résistante aux détergents (Detergeant resistant membrane, DRM), alors que dans le cas normal, cette protéine est également répartie entre DRM et la fraction membranaire soluble aux détergents (Sorek et al., 2007). Nous avons constaté que ROP6, contrairement à ROP2 ou ROP4, est nécessaire à l'accumulation de ROS induite par le signal osmotique et participe à certaines réponses des plantes au signal

osmotique. Le dépôt de lignine, la forme des cellules et la densité des racines latérales semblent être contrôlés par ROP6. Nous avons testé si ROP6 est suffisante pour déclencher la signalisation osmotique. Les lignées sur-exprimant GFP-ROP6 ne présentent aucune perturbation de leur signalisation, suggérant que l'activation de la ROP plutôt que la quantité de protéines pourrait être un facteur limitant. Pour tester cette hypothèse, nous avons utilisé des protéines mutées qui sont soit constitutivement actives (GTP-lock : ROP6-CA) ou constitutivement inactives (GDP-lock : ROP6-DN). L'expression transitoire dans des feuilles de tabac de capteurs à base de FRET (iROP) montre que ROP6-CA, contrairement à ROP6-DN, interagit avec le domaine CIRB de PAK1. Ce résultat confirme le comportement de verrouillage GTP ou GDP de, respectivement, ROP6-CA et ROP6-DN. Ainsi, des lignées complémentées avec la forme GTP-lock montrent une accumulation constitutive des ROS même en absence de stimulation. A l'inverse, les lignées exprimant la forme GDP-lock montrent une réponse atténuée. De plus, nous avons découvert que deux populations de molécules ROP6 coexistent dans la membrane plasmique et varient en fréquence quelques minutes après le traitement osmotique. Une analyse par microscopie de fluorescence à réflexion interne totale (TIRFM) de deux lignées transgéniques indépendantes a montré que la GFP-ROP6 a une localisation uniforme dans le PM dans des conditions contrôle. Par contre, lorsque les cellules sont soumises à un déficit hydrique moyen de -0,26 MPa et encore plus encore le cas d'un déficit sévère (-0,75 MPa), la GFP-ROP6 s'accumule en spots de taille réduite proches de la limite de diffraction.

Par conséquent, nous avons utilisé pour la suite le sptPALM, une technique d'imagerie à super-résolution récemment développée sur des échantillons de plantes (Hosy et al., 2015 mol plant).

En capturant par vidéo le déplacement de chaque molécule unique ROP6, deux comportements (hautement diffusible et faiblement diffusible) peuvent être observés en condition contrôle. Ce résultat montre que les molécules mEOS2-ROP6 diffusibles et relativement immobiles coexistent au sein des PM d'une seule cellule. Ensuite, nous avons examiné si les nanodomains contenant ROP6 sont impliqués dans la signalisation osmotique. Tout d'abord, en comparaison à la protéine ROP6 sauvage et en conditions témoin, ROP6-CA montre une proportion plus élevée de molécules immobiles et une plus grande fraction de molécules à haute densité locale. Par contre, aucune différence n'est observée entre ROP6 et ROP6-CA après traitement hyperosmotique, ce qui suggère que ROP6-CA est constitutivement associée aux nanodomains. D'autre part, il faut savoir que ROP6 est S-acylée transitoirement sur les cystéines 21 et 158. Ces modifications sont nécessaires pour localisation dans les membranes résistantes aux détergents et provoquent un retard de la diffusion latérale. Par contre, elles n'ont aucun impact sur l'activité ROP6 GTPase ou le ciblage PM. En utilisant le sptPALM, nous avons constaté que mEOS2-^{ROP6C21S / C158S} était insensible aux traitements par -0,75 MPa et que cette ROP6 mutée ne pouvait pas induire de ROS dans les cellules exposée au traitement hyperosmotique. Ces résultats montrent que la formation de nanodomains de ROP6 dans la membrane est nécessaire pour l'induction de la signalisation osmotique. Par FRET/FLIM, nous avons découvert que RbohD interagit préférentiellement avec la forme active de ROP6 et qu'une interaction avec ROP6 est observée lors d'une stimulation

osmotique. De plus, dans ces conditions, nous avons montré que ROP6 et RbohD coexistent dans les mêmes domaines de la membrane. Pour confirmer que les nanodomains RBOHD / ROP6 agissent comme une unité fonctionnelle pour la production de ROS dans la cellule végétale, nous avons testé si la formation de nanodomains ROP6 n'est pas une conséquence de la production de ROS. Ni l'inhibition de ROS par DPI / BPDS ni le traitement H_2O_2 n'a d'impact sur la formation du nanodomaine ROP6.

En parallèle de ce travail, il a été montré que ROP6 est nécessaire pour la réponse des cellules à la phytohormone auxine (Platre et al., 2019). Récemment, la formation de nanodomains ROP6, médiée par le lipide anionique phosphatidylsérine (PS), a été décrite en réponse à l'auxine. Cela suggère que la formation de nanodomains est une caractéristique générale de la voie de signalisation ROP6 chez les plantes. Nous avons examiné si l'agrégation (clustering) de RBOHD est également induite en réponse à la stimulation auxinique, comme cela se produit après induction de la voie de signalisation osmotique. Aucune augmentation de la densité des nanodomains de GFP-RBOHD n'a été observée dans de telles conditions. Ces résultats montrent que les nanoclusters de ROP6 formés après des stimulations auxinique ou osmotique peuvent différer dans leurs constituants qui, par conséquent, déterminent dans une certaine mesure la spécificité du signal.

Régulation de ROP6 par les GEF.

Comment un seul ROP peut répondre distinctement à plusieurs signaux est encore mal connu. Une hypothèse serait que le mécanisme d'activation en amont diffère selon les signaux. Comme le but ultime de ma thèse est de comprendre comment les plantes perçoivent et traduisent le signal osmotique, nous avons examiné les protéines qui pourraient venir en amont de ROP6 et agir comme des activateurs potentiels. En raison de leur rôle direct dans l'activation des ROP, nous nous sommes concentrés sur la famille des GEF, tout en gardant à l'esprit que l'activation des ROP peut également avoir lieu par inhibition des protéines de types GAP ou GDI. Les premiers candidats potentiellement intéressants de la famille des GEFs que nous avons testés sont GEF4 et GEF10 car ils interagiraient avec ROP6. De fait, le double mutant *gef4gef10* a des niveaux réduits de ROP activé, ce qui compromet l'initiation des poils racinaires. L'accumulation de ROS a été évaluée et un traitement osmotique induit une augmentation similaire des ROS, dans les plantes témoin (Col-0) et les lignées *gef4* et *gef10*. Outre GEF4 et GEF10, GEF2, GEF3, GEF11 et GEF14 sont également exprimés dans les mêmes types de cellules que ROP6. Nous avons testé des mutants uniques de perte de fonction *gef 2-1*, *gef3-1*, *gef1-1*, *gef14-1* et *gef14-2* dans des conditions contrôle ou de stress hyperosmotique. Cependant, une absence d'accumulation de ROS en réponse à un traitement osmotique n'a été observée que pour les deux allèles de perte de fonction de GEF14. Il semblerait donc que l'accumulation de ROS en réponse au signal osmotique soit dépendante de GEF14. Néanmoins, il reste possible qu'une certaine redondance puisse exister entre les isoformes, car les GEF sont connus pour former des hétéromères (Nagashima et al.2018). Nous avons donc testé des mutants arborant des pertes de fonction triples et quadruples, *gef 1/4/10* et *gef 1/4/10/14* (Waadt et Schroeder, 2016). Ces plantes poussent normalement, mais elles sont connues pour avoir une signalisation ABA altérée (Waadt et

Schroeder, 2016). Alors que pour le triple mutant *gef 1/4/10* on observe une induction normale des ROS, chez *gef 1/4/10/14* le traitement osmotique n'induit pas d'accumulation de ROS dans les cellules. Il semble donc que GEF14 est bien l'unique isoforme testée qui agit sur le signal osmotique. Comme expliqué précédemment, nous savons que ROP6 est impliquée dans des voies de signalisation autres qu'osmotique (Sorek et al., 2010, Poraty-Gavra et al., 2013, Wang et al., 2015, Platre et al., 2019, Smokvaska et al., 2020). Nous nous sommes donc demandés si GEF14 pourrait être impliquée dans ces autres voies de signalisation qui passent également par ROP6. Le mutant *gef14.1* a été incubé en présence de flg22 et ABA. Dans les deux cas, une augmentation du signal ROS a pu être détectée bien qu'elle soit légèrement inférieure dans le cas d'une stimulation par l'ABA. A titre de comparaison, d'autres mutants GEF ont été testés. Les mutants de GEF2 et GEF10 montrent une augmentation normale des ROS après stimulation avec la flg22 et l'ABA. Les données semblent indiquer que GEF14 est spécifiquement impliquée dans l'activation de ROP6 en condition de stimulation osmotique.

Interaction entre la signalisation du statut de la paroi et la signalisation osmotique

Comme expliqué dans l'introduction, le traitement osmotique a un effet direct sur le continuum paroi/membrane des cellules. Pour tester le rôle de la paroi cellulaire lors de la signalisation osmotique, nous avons décidé de tirer parti de l'isoxabène, qui inhibe le dépôt de cellulose en induisant la dissociation des complexes de dépôt CESA de la PM (Desprez et al., 2002; Tatenno et al., 2015). Les semis ont été traités avec 100 nM d'ISX pendant 2 h, et comparés avec un traitement par le le solvant de l'ISX, le diméthylsulfoxyde (DMSO). Le traitement de Col-0 par de l'ISX entraîne une suraccumulation des ROS induits par un choc osmotique, alors qu'aucun effet n'a été observé dans une situation de contrôle. Ce résultat suggère que les cellules traitées par ISX sont hypersensibles aux environnements osmotiques élevés. Nous avons également découvert que la perturbation de la biosynthèse de la cellulose a un effet sur la signalisation du stress osmotique. Ce résultat suggère que la communication entre la paroi et la cellule est important pour la signalisation osmotique. Afin de déterminer son rôle exact, nous avons étudié de manière extensive le rôle de la signalisation pariétale. Feronia (FER) est une kinase membranaire particulièrement intéressante. Effectivement, en plus d'être impliquée dans la signalisation pariétale des cellules, FER est primordiale dans de nombreuses voies de signalisation en réponse aux phytohormones (auxine, ABA, acide salicylique, ...) mais aussi aux stress mécanique et salin. De plus, il est connu que FER agit directement sur la famille des petites GTPase dont ROP6 fait partie.

Nos résultats ont montré que FER, mais aussi LRX3 qui fait partie du complexe de signalisation FER, sont nécessaires pour déclencher une accumulation de ROS induite par le signal osmotique. Mais, de manière surprenante, il semble que la kinase membranaire FER agisse en aval de ROP6. En effet, la mutation du gène *FER* supprime totalement l'accumulation de ROS induite par la forme autoactive de ROP6, et ce, sans qu'aucun effet sur la quantité de protéine ou sa localisation ne soit observé. Comme nous n'avons pas observé de phénotype évident sur la localisation ROP6-CA, nous avons émis l'hypothèse que FER pourrait réguler l'organisation spatiale de ROP6 au sein de la membrane. De fait, des travaux de nos collaborateurs à Lyon ont montré que la composition et l'organisation de la phosphatidylserine (PS) sont essentielles pour

permettre la signalisation ROP6 (Platre et al., 2019). Pour tester un lien potentiel entre les nanodomains contenant ROP6, la PS et FER, nous avons d'abord testé le rôle du PS lors de la signalisation osmotique. Le mutant *pss1-3*, qui n'a pas de PS, ne montre aucune accumulation de ROS en réponse au signal osmotique (Platre et al., 2018). De son côté, la lignée PSS1-OX, qui a deux tiers de PS en plus, n'a pas révélé d'augmentation supplémentaire de l'accumulation de ROS. Ces résultats montrent que les PS sont nécessaires mais non limitantes pendant la signalisation osmotique. Comme les deux allèles mutants de FER ne présentent pas d'altération de la quantité de PS, nous avons émis l'hypothèse que la localisation des PS serait affectée chez *fer*. Nous avons utilisé deux biosenseurs de PS bien décrits, 2xPH^{EVECTIN} et C2^{LACT} (Platre et al., 2018, Simon et al., 2016) et nous les avons introgressés dans un fond mutant *fer4* ou *fer5*. Nous avons observé une diminution du signal présent à la PM des deux biosenseurs de PS. Nous avons également découvert que les nanodomains PS agissent en aval de la signalisation FER. Effectivement, il est connu qu'il est possible de compléter les phénotypes du mutant de synthèse de PS (*pss3-1*) par un ajout exogène de Lyso-phosphatidylserine (LPS). Les racines de *fer4x2xPH^{EVECTIN}* et *fer4xC2^{LACT}* supplémentées pendant 1 heure avec du LPS, mais pas avec du Lyso-acide phosphatique (LPA), montrent une relocalisation des biosenseurs PS vers la PM, les rendant en tout point identiques avec les racines de plantes de type sauvage. Comme il est possible de compléter la localisation des PS par un ajout de LPS dans le milieu, nous avons voulu tester si cet effet permettait de récupérer la signalisation osmotique en aval de ROP6. Un traitement exogène avec du LPS peut, d'une part, restaurer la production de ROS induite par le signal osmotique chez les deux allèles mutants de FER. Ce traitement complète également le phénotype des plantes *fer4xmCit-ROP6gCA* et *fer5xmCit-ROP6gCA*. Par contre, la perte d'accumulation de ROS induite par le signal osmotique observée chez les plantes *rop6.2* ne peut pas être reversée par le traitement LPS. Ces données indiquent que PS agit bien en aval de l'activation de ROP6 et surtout que FER régule la quantité de PS à la membrane, qui, à son tour, agit sur la signalisation ROP6. Nous avons ensuite cherché à déterminer si ce défaut pouvait expliquer certains des autres phénotypes induits par la perte de fonction de FER. Par exemple, *fer4* a un défaut dans l'organisation de son épiderme foliaire. Ce défaut dans la forme des cellules est très vraisemblablement sous le contrôle de la signalisation des petites GTPases. En cultivant des plantules *fer4* pendant 3 jours sur des milieux contenant du LPS, nous avons observé la restauration de la forme de cellules de l'épiderme foliaire. Cette complémentation n'est pas visible lors du traitement de *fer4* par du LPA. Ces résultats suggèrent que la régulation de la localisation des PS par FER est un déterminant important pour plusieurs voies de signalisation et de développement des plantes. Comme expliqué dans l'introduction, la voie de détection de l'état de la paroi cellulaire est médiée par différents CrRLK1L. Nous avons donc cherché à déterminer si d'autres CrRLKs pouvaient avoir un effet similaire sur la localisation des PS. Parmi eux, THESEUS apparaît central pour la régulation des réponses de croissance cellulaire rapide. Nous avons utilisé un allèle perte de fonction (*the1-1*) (Hematy et al., 2007) et un allèle gain de fonction *the1-4* (Mertz et al., 2017) incubés en conditions contrôles ou en présence d'une solution à -0,75 MPa. Par rapport à Col-0, le mutant perte de fonction (*the1-1*) ne montre pas

d'augmentation de ROS en réponse à un stress osmotique. Par contre, le mutant gain de fonction (*the1-4*) montre un phénotype de type Col-0. Ces résultats indiquent que le bon fonctionnement de la signalisation du statut de la paroi via THE1 est nécessaire pour l'induction osmotique de ROS. Nous avons également pu montrer que ce phénotype peut être complété par ajout exogène de LPS, suggérant que cette CrRLK agirait de manière similaire à FER sur la composition en PS de la membrane.

Discussion générale

Au cours de mes travaux, nous avons identifié de nouveaux acteurs moléculaires dans la cascade de signalisation osmotique. En partant de l'accumulation de ROS en réponse à un stimulus hyperosmotique, nous avons pu décrire qu'il existe deux voies majeures pour déclencher une production de ROS. En amont de celles-ci, une Rho GTPase, ROP6, exerce un rôle régulateur essentiel. Nous avons également découvert un activateur de ROP6, GEF14 qui régule spécifiquement ROP6 en réponse à des stimuli osmotiques, mais non en réponse à d'autres signaux (tels que ABA ou flg22). De plus, la présence de phosphatidylsérine (PS) organisée en cluster est nécessaire pour assurer une signalisation ROP6 normale. L'organisation des PS dans la membrane plasmique est régulée par la CrRLK, FER, qui, selon nous, coordonne la signalisation GTPase en général. Dès lors, il semble que la détection de l'état de la paroi cellulaire par FER ne soit pas une conséquence de la signalisation osmotique mais plutôt une signalisation conjointe agissant comme un rhéostat.

De plus, ce travail de thèse a mis en évidence que ROP6 est importante pour l'organisation de Rboh dans la MP lors dans la signalisation osmotique. Effectivement, nous avons montré que la forme autoactive de ROP6 interagit constitutivement avec RBOHD, co-localise dans les nanodomains de ROP6 et induit une production de ROS, même en absence de stimulation. A l'inverse, lorsque ROP6 ne peut pas s'accumuler dans les nanodomains, on n'observe aucune production de ROS même après un traitement hyperosmotique. Néanmoins, nous avons aussi montré que RBOHD forme des clusters même en absence de ROP6, démontrant que l'organisation en nanodomains de RBOH n'est pas suffisante pour induire la production de ROS. Il est probable que d'autres composants sont également nécessaires. Durant la signalisation osmotique, il est possible que RbohD soit dirigée vers les nanodomains grâce à ROP6, nanodomains dans lesquels elle va retrouver RbohF qui semble être pré-organisée en clusters. Notons quand même qu'avec notre système, nous ne pouvons pas dire avec certitude que RbohD et RbohF sont les mêmes clusters. Cette hypothèse supposerait que les Rboh forment des hétéromères. Des résultats basés sur les ROS induits par le signal osmotique dans les doubles et les simples mutants de RbohD et RbohF indiquent une interaction génétique (Martiniere et al., 2019). Le changement de localisation des Rboh dans le PM pourrait être un déclencheur pour d'autres régulateurs tels que BIK1 et/ou les CPKs. Ces régulateurs pourraient également changer la localisation de Rboh. Il n'est pas exclu que certains d'entre eux puissent résider dans le même cluster que ROP6 / Rboh permettant de moduler l'état de phosphorylation de Rboh.

Les propriétés uniques de la ségrégation des protéines dans les nanodomains à l'intérieur de la membrane apparaissent comme une caractéristique essentielle dans

plusieurs voies de signalisation. J'ai décrit que lors d'un stress hyperosmotique, la ROP6 co-ségrègeait avec son effecteur Rboh dans des nanodomains de la PM. Parce que ROP6 est également ciblée dans les nanodomains de manière dépendante du PS dans un contexte de signalisation auxinique (Platre et al., 2019), notre hypothèse est que plusieurs domaines existent en fonction du signal amont et que les constituants du domaine contribuent à la spécificité du signal. Le nanopatterning de ROP6 suit un comportement de type interrupteur (hors du cluster au repos, à l'intérieur du cluster en état activé). Par conséquent, ROP6 ne rencontrerait ses effecteurs que lorsqu'elle est présente dans des nanodomains. En effet, après un signal osmotique mais pas auxinique, Rboh est recrutée dans les même nanodomains que ROP6. Sur le feuillet externe de la membrane plasmique des cellules végétales, les GIPC (glycosylinositolphosphoryl-céramides) sont les lipides les plus abondants (Gronnier a et al., 2016). Ils ont de grandes chaînes lipophiles qui pourraient connecter les deux feuillets de la membrane plasmique. Surtout, ils possèdent une tête chargée qui pourrait interagir avec les charges fixes de la paroi. On pourrait supposer que ce système permet la formation de nanodomains PS stables dans le feuillet de la membrane plasmique cytosolique par couplage avec les GIPC. Ainsi, il serait possible de « mimer » la structuration de la paroi avec celle de la membrane plasmique.

La protéine GEF14 constitue un candidat très prometteur car elle est nécessaire à l'accumulation des ROS induites osmotiquement. De plus, GEF14 pourrait être le facteur responsable de l'activation de ROP6 spécifiquement lors de la signalisation osmotique. Une étude d'interaction appropriée sera nécessaire pour confirmer l'interaction réelle entre ROP6 et GEF14 et, surtout, nous devons savoir si GEF14 agit en amont de ROP6. La découverte que GEF14 pourrait être l'activateur en amont de ROP6 dans la signalisation osmotique pourrait être une caractéristique intéressante pour essayer de mieux comprendre les nanodomains spécifiques dans les plantes. En fonction des stimuli environnementaux, ABA, osmotique, auxine, flg22, différentes GEF activeraient ROP6, entraînant son recrutement dans un type spécifique de nanodomains.

Nos données indiquent qu'un récepteur kinase FERONIA pourrait réguler la localisation de PS et par cela réguler probablement la signalisation ROP. Toutefois, le mécanisme par lequel ce processus se produit n'est toujours pas clair. Une manière de l'expliquer est de relier la régulation FER et l'adressage des PS à la PM. Nous pouvons émettre l'hypothèse que FER pourrait réguler l'activité de certaines protéines de transfert lipidique et donc réguler le contre-transport PS- PI4P dans les plantes (même si ce mécanisme n'est pas encore décrit chez les plantes). Cela pourrait être réalisé grâce à l'activité kinase de FER qui phosphorylerait des protéines impliquées dans le ciblage des PS à la membrane PM. Alternativement, FER agirait par son activité d'échafaudage, comme c'est le cas pour la signalisation pathogène PTI (Stegmann et al., 2019).

Enfin, deux processus cellulaires majeurs tels que la détection du statut de la paroi et la signalisation osmotique partagent des acteurs moléculaires communs mais conduisent à des réponses différentes. Qu'est-ce que cela pourrait bien signifier pour une cellule? L'allongement des cellules est soigneusement contrôlé par la machinerie de maintenance de l'intégrité de la paroi cellulaire. Comme le changement extracellulaire d'osmolarité impacte directement la turgescence, une cellule en élancement doit être plus sensible au

changement extérieur d'osmolarité, de concentration ionique ou même à plus grande échelle, de la disponibilité en eau. C'est peut-être la raison pour laquelle ces deux processus nécessitent une coordination très fine. Pour conclure, le nanoclustering de ROP peut avoir plusieurs fonctions critiques pour la signalisation. Nous proposons en particulier une compartimentation du signal entre différentes ROP qui ont par ailleurs des séquences très similaires et qui se lient à des effecteurs similaires *in vitro*, mais pas *in vivo*. La formation de nanoclusters ROP pourrait donc être un outil pour générer à la fois une haute-fidélité et une grande spécificité pendant la transduction des signaux cellulaires. L'utilisation de l'imagerie à haute résolution développée pendant ma thèse permettra à l'avenir de tester ces différents concepts et d'interroger leur importance dans le développement et la physiologie des plantes.